EFFECTS OF BINARY MIXTURES OF XENOESTROGENS ON GONADAL DEVELOPMENT AND REPRODUCTION IN ZEBRAFISH

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ABSTRACT

Previous studies exposing fish to xenoestrogens have demonstrated vitellogenin (VTG) induction, delayed gametogenesis, altered sex distribution, and decreased reproductive performance, with a majority of those studies focusing on exposure to single chemicals. The objective of this study was to determine the effects of binary mixtures of a weak estrogen receptor agonist, nonylphenol (NP) and a potent estrogen receptor agonist, 17α-ethinylestradiol (EE) on sex distribution, gametogenesis, VTG induction, heat shock protein 70 (HSP70) expression and reproductive capacity in zebrafish (*Danio rerio*). Fish were exposed from 2 to 60 days post-hatch (dph) to nominal concentrations of 10 or 100 µg/l NP (NP10 or NP100, respectively), 1 or 10 ng/l EE (EE1 or EE10, respectively), 1 ng/l EE + 10 or 100 µg/l NP (EE1+NP10 or EE1+NP100, respectively), 10 ng/l EE + 10 or 100 µg/l NP (EE10+NP10 or EE10+NP100, respectively) or solvent control (0.01% acetone v/v) in a static-renewal system with replacement every 48h. At 60 dph, fish from each treatment were euthanized for histological examination of gonads, and whole body VTG and HSP70 levels. Remaining fish were reared in clean water until adulthood (240 dph) for breeding studies. In all EE10 exposure groups (EE10, EE10+NP10 and EE10+NP100), increasing NP concentration acted less than additively to the action of EE in terms of VTG induction at 60 dph. Similarly, a less than additivity of effect was observed with egg production, where EE1+NP100 exposure resulted in significantly more eggs produced per breeding trial than EE1 alone. Histological staging of oogenesis revealed suppressed gametogenesis in females at 60 dph. There were no differences among treatment groups in whole body HSP70 expression in 60 dph fish or in gonadal HSP70 expression in adult fish. Although there was no statistical evidence of non-additivity, breeding trials in adults revealed significant reductions in egg viability, egg hatchability and/or F_1 swim-up success, suggesting that developmental exposures to xenoestrogens may cause irreversible

effects on egg quality and progeny even after depuration. In conclusion, these results suggest that environmentally relevant mixtures of NP and EE can produce additive or non-additive effects depending on the particular response being determined and the respective exposure concentrations of each chemical. Thus, it is recommended that caution be exercised in ecological risk assessments when assuming additivity in piscine responses to xenoestrogen mixtures.

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ACTH	Adrenocorticotropic hormone
AEBSF	2-aminoethyl-benzenesulfonyl fluoride
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
APnEO	Alkylphenol polyethoxylate
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
Dph	Days post-hatch
E2	17β-estradiol
EDC	Endocrine disrupting chemical
EE	17α-ethinylestradiol
ELISA	Enzyme linked immunosorbent assay
EO	Ethylene glycol
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GtH	Gonadotropin
GSI	Gonadosomatic index
HPLC	High pressure liquid chromatography
HSI	Hepatosomatic Index
HSP70	Heat shock protein 70
LH	Luteinizing hormone
m	Meta-substituted
NBT	Nitro-blue tetrazolium chloride
NP	Nonylphenol
NPEO	Nonylphenol polyethoxylates
o	Ortho-substituted
OD	Optical density
OPD	*O*-Phenylenediamine
p	Para-substituted
PCB	Polychlorinated biphenyl
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
S/E	Swim-up to fertilized egg ratio
S/H	Swim-up to hatch ratio
TSH	Thyroid-stimulating hormone
T3	Triiodothyronine
T4	Thyroxine
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal dideoxynucleotidyl transferase dUTP nick end labeling
VTG	Vitellogenin

CHAPTER 1
INTRODUCTION

1.1 Vertebrate Endocrine System

In humans and other vertebrates, many physiological systems within the organism interact closely with each other to maintain a homeostatic environment in order to achieve functional healthy individuals. Some of the more noteworthy of these include the central nervous, cardiovascular, immune and endocrine systems. The endocrine system is particularly important as it regulates many of the body's functions, including growth and development, metabolism, regulation of the internal environment (temperature, water balance, ions), and reproduction. The endocrine system is a complex network of specialized cells and glands that behaves as a communication system by releasing hormones, the natural chemical messengers, from endocrine glands into the bloodstream in response to stimuli. Endocrine glands are ductless glands; major examples include the pituitary, pineal, thyroid and adrenal glands, pancreas, as well as the reproductive glands; ovaries and testes. Other non-endocrine organs - such as the brain, heart, lungs, kidneys, liver, thymus, skin, and placenta - also produce and release hormones. Hormones act on their target cells by controlling one of the three following processes: (1) the rates of enzymatic reactions, (2) the transport of molecules across cell membranes, or (3) gene expression and protein synthesis (Silverthorn, 1988).

The endocrine system works in close relation with the nervous system, particularly the hypothalamus. The hypothalamus, located in the lower central part of the brain, is composed of specialized cells and functions as the main link between the endocrine and nervous systems. The hypothalamus controls the pituitary gland through the production and discharge of releasing

hormones from hypothalamic nerve cells that either stimulate or suppress hormone secretions from the pituitary. Examples of releasing hormones include thyrotropin-releasing hormone, corticotropin-releasing hormone, gonadotropin-releasing hormone and growth hormone releasing hormone (Silverthorn, 1988).

The pituitary is often called the "master gland" as it produces hormones that control several other endocrine glands. Examples of pituitary hormones include growth hormone, thyroid-stimulating hormone, adrenocorticotropic hormone, prolactin and gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) (Silverthorn, 1988).

Among the pituitary hormones, FSH and LH are of particular importance due to their involvement in reproduction. In most fish species studied to date, there are two gonadotropins, termed GtH I and GtH II, which are analogous to mammalian FSH and LH, respectively (Swanson et al., 1991). Gonadotropin I is involved in gametogenesis and steroidogenesis, while GtH II is involved in the final maturation stages of gametogenesis. Similar to other vertebrates, these piscine gonadotropins stimulate the production of sex steroids, which then act on target tissues to regulate processes such as gametogenesis, reproduction, sexual phenotype, and behaviour (Arcand-Hoy and Benson, 1998). The link between the hypothalamus, pituitary and gonad in vertebrates has been termed the hypothalamic-pituitary-gonadal axis and is under the influence of feedback mechanisms (Figure 1.1). For instance, ovary-produced estrogen can affect the hypothalamus positively (upregulation) or negatively (downregulation), depending on the current need of the hormone necessary to fulfill the physiologic and reproductive requirements of the fish (Arcand-Hoy and Benson, 1998). Consequently, any disruptions in the hypothalamus-pituitary-gonadal axis that cause hormone level changes could potentially lead to reproductive impairment.

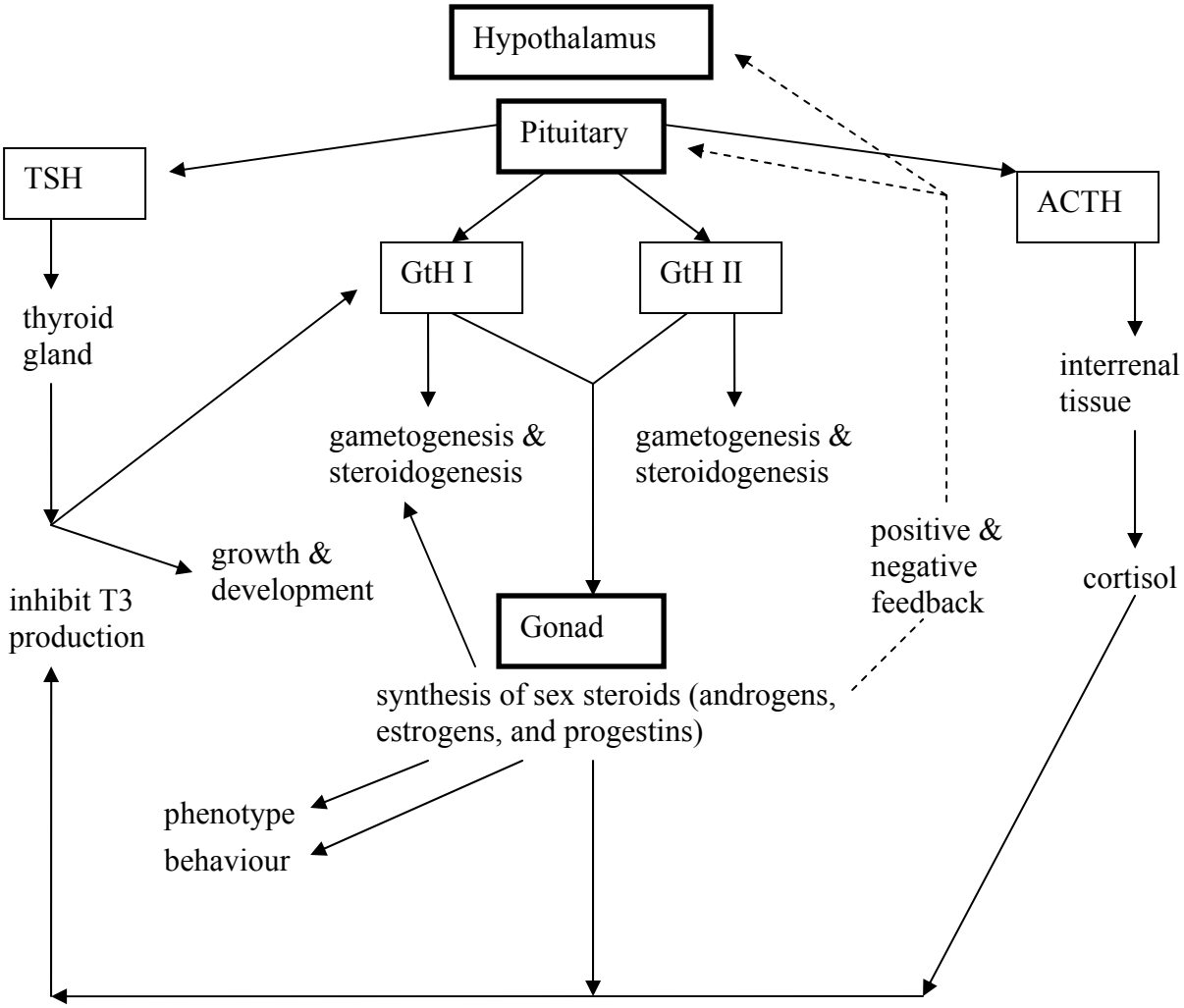


Figure 1.1 Interrelationship of the piscine hypothalamic-pituitary-gonadal axis. The synthesis and release of sex steroid hormones, thyroid hormones, and corticosteroid hormones are under the control of the hypothalamus and pituitary. The gonadotropins (GtH I, GtH II) both have an influence on the synthesis of sex steroids, which in turn regulate reproduction, phenotype, and behavior. The thyroxine (T4) to triiodiothyronine (T3) conversion may be inhibited by elevated plasma sex steroids and cortisol. The effect of gonadotropin-mediated events such as oocyte development and maturation may be enhanced by the presence of T4. The hypothalamic-pituitary-gonadal axis is ultimately controlled by feedback systems (dotted-line). TSH, thyroid-stimulating hormone; GtH I, gonadotropin I; GtH II, gonadotropin II; ACTH, adrenocorticotropic hormone. (Reproduced and adapted from Arcand-Hoy and Benson, 1998)

1.2 Sources and Types of Xenoestrogens

Increasing concerns have arisen over chemicals found in the environment which are able to impair sexual differentiation and other hormone regulated processes by mimicking or blocking the action of natural hormones, potentially causing adverse human and ecological health effects. Because of their ability to imitate, inhibit, or alter the activity of endogenous hormones, thereby interfering with normal endocrine system functions, they have been aptly termed "endocrine disrupting chemicals" (EDC). Sources of EDCs vary widely, ranging from the manufacturing and disposal of industrial and agricultural chemicals to medical and household products (Daughton and Ternes, 1999). Some EDCs are released into the environment inadvertently, as in the case of industrial byproducts and discharges, while others, such as pesticides, are released intentionally into the environment in controlled amounts. For pharmaceuticals, household chemicals, other consumables and biogenic hormones, they are usually released into the environment after passing through sewage treatment plants, which generally are not equipped to remove them from the effluent (Halling-Sorensen et al., 1998). Examples of chemicals with known endocrine disrupting property include certain pesticides, phthalates, phytoestrogens, and pharmaceuticals (Kavlock et al., 1996; Tyler et al., 1998).

Presently, estrogenic EDCs, also known as xenoestrogens, are receiving the most attention after speculation that they may contribute towards the observed increased incidence of human reproductive disorders. Examples include testicular cancer, cryptorchidism (one or both testicles that have not descended), hypospadias (displacement of urethral opening) and decreased sperm counts in males as well as breast cancers in females; however, no conclusive evidence has been obtained so far to support this hypothesis (Rivas et al., 1997; Allen et al., 1999; Safe, 2000). Nevertheless, studies conducted on wildlife species of fish, birds, reptiles and mammals have shown various degrees of physiological as well as histological alterations. The observed effects

include decreased reproductive fitness, demasculinization, feminization, decreased hatchability of eggs, modification of the immune system, and increased cancer incidence (Colborn et al., 1993; Fry, 1995; Kavlock et al., 1996; Tyler et al., 1998). For example, in Lake Apopka, Florida, an aquatic environment known to have been contaminated with a spill of organochlorine pesticides from a chemical waste site, increased plasma 17β-estradiol (E2) concentration and abnormal ovarian morphology were observed in female alligators (*Alligator mississippiensis*), while abnormal germ cells in testes, reduced penis size and lowered testosterone levels were detected in male alligators (Guillette et al., 1994; Guillette et al., 1995; Guillette et al., 1996). Meanwhile, feminization of gulls has been linked to exposure to DDT and DDE, organochlorine pesticides known to induce estrogenic effects (Fry and Toone, 1981). In marine gastropod species, imposex (females possessing male characteristics) has been associated with exposure to tributyltin, the active ingredient used in antifouling paints in marine vehicles (Ellis and Pattisina, 1990). In fish, where a great number of recent studies have focused, estrogenic exposures led to changes in plasma hormone concentrations (Khan and Thomas, 1998) and gonadal size (Jobling et al., 1996; Ashfield et al., 1998), development of ovotestis (gonad that contains both ovarian follicles and testicular tubular elements) (Gray and Metcalfe, 1997) as well as induction of vitellogenin (VTG), an egg yolk precursor normally found in the blood of maturing females, in male fish (Jobling et al., 1996).

Based on their origin, xenoestrogens can be categorized into four broad groups; natural steroidal, synthetics, phytoestrogens and other estrogenic industrial compounds. Examples of natural steroidal estrogens include the endogenous vertebrate hormone E2 and its metabolites, estrone and estriol. Diethylstilbestrol and 17α-ethinylestradiol (EE) are good representatives of synthetic estrogens. Phytoestrogens are botanically derived, such as β-sitosterol, found in many

plants including rice bran, wheat germ, corn oils, and soybeans. Lastly, estrogenic industrial compounds include a wide variety of chemicals whose estrogenicity is often uncovered inadvertently, for instance 4-nonylphenol (NP). NP was discovered to leach from the polystyrene containers used during the development of a screening assay for estrogenic compounds (Soto et al., 1991). The estrogenic potencies of these chemicals vary greatly and differences between some of them can reach several orders of magnitude (MacLatchy et al., 1997; Mazur and Adlercreutz, 1998; Islinger et al., 1999; Servos, 1999; Lindholst et al., 2000). Overall, due to natural and synthetic estrogens' stronger affinity towards the estrogen receptor (ER), they are much more potent than phytoestrogens and estrogenic industrial compounds, however, environmental concentrations of phytoestrogens and estrogenic industrial compounds are generally much higher than that of natural and synthetic estrogens (Spengler et al., 2001).

Xenoestrogens can also be divided, based on their mode of action, into receptor agonists and antagonists. Agonists mimic the action of endogenous hormones, inducing receptor-mediated responses while antagonists act oppositely and block the responses of the same pathway (Danzo, 1997; Cheek and McLachlan, 1998). The lipophilicity of some xenoestrogens also allows trans-generational toxicity to occur when the chemicals are transferred maternally to their offspring. Polychlorinated biphenyls have been demonstrated to accumulate in the oocytes of female fish, which could lead to subsequent exposure of their progeny to these compounds (Olsson et al., 1999; Metcalfe et al., 2000).

Although xenoestrogens come from a wide array of chemical classes, most do share a structural commonality of having phenol ring(s) which allows them to be highly lipophilic, highly bioaccumulative, and have long half-lives (Danzo, 1997). These characteristics allow

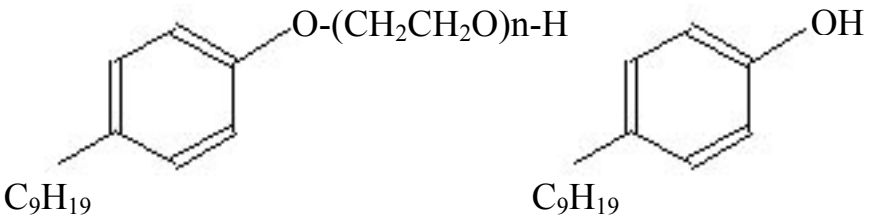
them to accumulate in fatty tissues of organisms and persist in the environment (McLachlan et al., 1984).

Another common feature shared amongst xenoestrogens is their ability to bind to ERs, though each bind with differing affinity. Most environmental estrogens bind to ERs with an affinity at least three (Sumpter and Jobling, 1995), and typically four to six (Lee and Peart, 1998) orders of magnitude less than the endogenous estrogen, E2. However, because of their ability to bioaccumulate and persist in the environment, relatively weak estrogenic compounds may accumulate to a high enough concentrations to induce estrogen-mediated responses.

Wastewater from sewage treatment plants is a common and major source of xenoestrogens, including NP and EE in the USA (Kolpin et al., 2002). Many studies have demonstrated that sewage effluents are estrogenic to fish (Purdom et al., 1994; Larsson et al., 1999; Svenson et al., 2002). Nonylphenol is a degradation product of nonylphenol polyethoxylates of the alkylphenol polyethoxylate (APnEO) family. APnEO are North America's second largest class of nonionic detergents in commercial production (Naylor et al., 1992; White et al., 1994). Their broad applications include the manufacturing of plastics, pesticides, herbicides, paints, cosmetics and cleaning products (Talmage, 1994). Worldwide APnEO production has been estimated to be >0.3 Mt yr^{-1} (White et al., 1994) with 0.2Mt yr^{-1} in the United States (Ahel and Giger, 1985; Naylor et al., 1992). One of the reasons why APnEOs and their degradation products have received much concern and research interest is their environmental persistence, as they have been identified in relatively high concentrations in industrial sewage effluents and in sediments in lakes and rivers in Europe (Jobling and Sumpter, 1993; Sumpter and Jobling, 1995; Jobling et al., 1996; Nimrod and Benson, 1996).

1.2.1 Xenoestrogen: Nonylphenol

Nonylphenol polyethoxylates (NPEOs) are the most extensively used of the APnEO family. The annual production of NPEOs from the US, Western Europe, and Japan totaled around 0.35 Mt (Ahel et al., 1994). In 1989, NPEO usage in Canada was 6.0 kt, with the number expected to rise with time (Bennie et al., 1998). Their general structure consist of a phenol core connected to one of many hydrophobic, branched, isomeric nonyl moieties via o, m, or p substitution, and a hydrophilic ethoxylate chain ether linked at the phenolic oxygen (John and White, 1998) (structure detailed in Figure 1.2). The s in NPEOs denotes the number of ethylene glycol (EO) units present in the molecule with NP representing nonylphenol (John and White, 1998). Nonylphenol polyethoxylates themselves do not exhibit any estrogenic activity. However, during wastewater treatment, they are biodegraded to shorter homologs; ones containing fewer number of EO units, which are estrogenically active and eventually discharged into the aquatic environment. The first stage of NPEO biodegradation is relatively efficient. In this process, *Pseudomonas putida*, a strain of bacteria normally found in the activated sludge portion of the wastewater treatment system, drives the progressive conversion of parent NPEOs into short-chain ethoxylates, such as 4-nonylphenoxyacetic acid and 4-nonylphenol diethoxylate via hydrolytic removal of EO on the NPEOs (Field and Reed, 1996; John and White, 1998; Bennie, 1999). A further degradation process of the resultant compounds to NP, the fully deethoxylated form, occurs in anaerobically stabilized sewage sludge (Bennett and Metcalfe, 1998). Nonylphenol and lower homologs of NPEOs are more persistent and lipophilic than the parent NPEOs, thus the level of toxicity increases as the biodegradation process proceeds (Ahel et al., 1994; Liber et al., 1999; Lye et al., 1999; Maguire, 1999).



Nonylphenol polyethoxylate (NPE) Nonylphenol (NP)

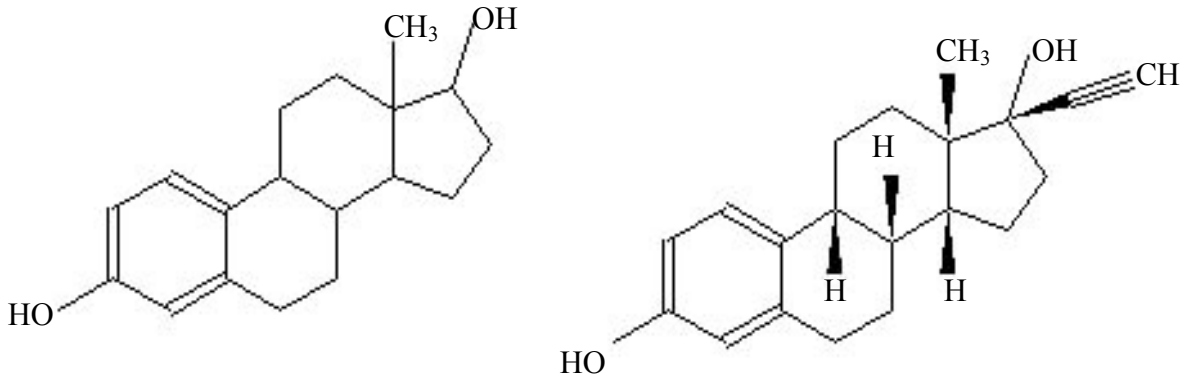
Figure 1.2 Chemical structures of nonylphenol polyethoxylate and nonylphenol.

Recent research has identified NP as the most significant degradation product of NPEO because of its estrogenicity, toxicity, enhanced resistance toward biodegradation, and bioaccumulation in aquatic organisms (Talmage, 1994; Bennie, 1999; Liber et al., 1999). Studies conducted on several fish species have shown NP to induce development of ovotestes while delaying testicular development and inhibiting spermatogenesis in males, cause increased levels of VTG and zona radiata protein in both males and females, and altered gonadosomatic indices (Jobling et al., 1996; Gray and Metcalfe, 1997; Arukwe et al., 1998; Arukwe et al., 2000; Kinnberg et al., 2000). In Atlantic Canada, the use of NP as a solvent/emulsifier in pesticide spraying has been implicated for the declining Atlantic salmon population (Fairchild et al., 1999). In the U.S., a study conducted by the Alkylphenol & Ethoxylates Program Panel of the U.S. Chemical Manufacturers Association found great variability between measurements of environmental concentrations of NP from different sample sites. In sites downstream of industrial or wastewater treatment facilities, the mean NP concentrations in water was 0.12 μg/l with the maximum concentration observed being 64 μg/l. Sixty percent of the test sites showed less than detectable NP levels (Talmage, 1994). The amount of NP in the sediments of the same bodies of water showed much variability with measurements between 2.9-2960 μg/kg, with a

mean of 161.9 μg/kg (Talmage, 1994). NP levels in receiving waters of sewage treatment facilities measured in the range of 325-1000 μg/l (Talmage, 1994).

1.2.2 Xenoestrogen: 17α-ethinylestradiol

Another common xenoestrogen found in sewage treatment plants is EE (structure detailed in Figure 1.3); a potent synthetic estrogen and one of the most commonly used active ingredients in oral contraceptives (Purdom et al., 1994). The majority of EE is excreted from humans as glucuronide conjugates, some of which revert back to the parent EE via microbial β-glucuronidase activity during wastewater treatment (Desbrow et al., 1998). Measurements taken from sewage treatment plant effluents around the world report EE concentrations ranging from non-detectable to 0.38 ng/l in the rivers Thames, Calder, and Aire, UK (Williams et al., 1999), non-detectable to 7.0 ng/l (Desbrow et al., 1998), and non-detectable to 42 ng/l at sewage treatment plants in Ontario, Canada (Ternes et al., 1999). Similar to NP, in fishes exposed to EE, increased VTG production along with reduction in gonadosomatic index were evident in males and females, as well as alterations in female gonadal physiology, and altered testis histology in males (Van den Belt et al., 2002). Of particular concern is the exposure to xenoestrogens or other EDCs during early developmental stages since many of the endocrine feedback mechanisms that are present in adults may not be fully developed in juveniles. Consequently, adverse effects may be observed at doses lower than those seen in adults.



17β-estradiol 17α-ethinylestardiol

Figure 1.3 Chemical structures of the endogenous estrogen 17β-estradiol (E2) and synthetic estrogen 17α-ethinylestradiol (EE).

1.3 Target Receptor: Estrogen Receptor

As aforementioned, one commonality amongst xenoestrogens is the ability to interact with ERs, which are found either at the cell surface or the cell nucleus of various target tissues. Nuclear ERs are ligand-dependent transcription factors belonging to the steroid/thyroid/retinoic acid superfamily. All members of this family share a basic structure consisting of six domains (A-F). These are the variable N-terminal domain (A/B), a highly conserved zinc-finger containing domain that binds DNA (C or DBD), a variable hinge region (D), a well-conserved ligand binding domain (E or LBD), and a variable C-terminal region (F). When ligand binds to the receptor, the conformation of the LBD is changed, inducing receptor dimerization. Subsequently, the receptor-ligand dimer interacts with specific sequences in the target gene, modulating its transcription (Hewitt and Korach, 2002).

In mammals, two ER subtypes, ERα and ERβ have been identified. In fish, piscine ERs share a number of commonalities with their mammalian homologues, with two major

differences. First, in addition to the full-length ERα, an isoform lacking the A domain and containing a ligand-independent transactivation function has been isolated in fish as well as in other oviparous species. This isoform is mainly, if not solely, expressed in the liver and is believed to play a role in hepatic vitellogenesis (Melamed and Sherwood, 2005). Secondly, recent research have demonstrated that most teleost fish, including the zebrafish (*Danio rerio*), possess two distinct forms of ERβ, ERβa and ERβb, encoded by two distinct genes (Hawkins and Thomas, 2004).

1.4 Biochemical Indicators of Xenoestrogen Exposure and Effect

1.4.1 Vitellogenin

Induction of protein transcription and translation is one of the most studied responses to xenoestrogen exposures; specifically, vitellogenin (VTG) and zona radiata (eggshell) proteins in oviparous vertebrates have gained wide acceptance as reliable biomarkers for xenoestrogen exposures (Tyler et al., 1996; Arukwe et al., 1997b).

Vitellogenin is a calcium-binding phospholipoglycoprotein vital in the reproduction process of oviparous vertebrates. Its classification as a phospholipoglycoprotein indicates the important functional groups that it possesses; lipids, several carbohydrates, and phosphate groups (Mommsen and Walsh, 1988; Silversand and Haux, 1995). As well, the maturing oocytes receive most of its mineral supplies as a result of the ion-binding capability of VTG. Produced in the liver, VTG is synthesized and released under hormonal regulation through the hypothalamic-pituitary-gonadal-liver axis. 17β-estradiol, the endogenous estrogen, is the primary hormone that stimulates the release of VTG from the liver into the blood. It then traverses to the ovaries where it is cleaved into phosvitin and lipovitellin, which are incorporated into the yolk of growing oocytes as a source of nutrients for embryos (Mommsen and Walsh, 1988; Nicholas, 1999). In sexually maturing female fish, VTG levels rise gradually, and in some

species plasma concentrations can reach several milligrams per milliliter (Copeland et al., 1986; Tyler et al., 1996). In contrast, VTG concentration is very low or absent in male and juvenile fish, but can be induced upon xenoestrogen exposure (Sumpter and Jobling, 1995; Folmar et al., 1996; Jobling et al., 1996; Tyler et al., 1998). It is important to note that although males do carry the VTG gene, endogenous E2 levels in male fish are usually too low to induce the production of VTG (Flouriot et al., 1993).

1.4.2 Heat Shock Protein 70 (HSP70) and Apoptosis

Although useful as a biomarker of exposure, VTG induction does not offer much insight regarding the cause(s) of observed adverse effects associated with xenoestrogen exposures. Recent studies have suggested that apoptosis and heat shock protein 70 (HSP70) induction may be involved with the observed toxicity of estrogenic compounds and other toxicants (Janz et al., 1997; Weber and Janz, 2001; Weber et al., 2002a; Weber et al., 2002b; Yoo and Janz, 2003). Thus, further investigation on the relationships between apoptosis, HSP70 induction, and estrogenic toxicity is warranted towards a better understanding of how estrogenic compounds are affecting the fish reproductive system.

Apoptosis is a physiological process whereby cells are induced to die; i.e. programmed cell death. It is thought that apoptosis can act as an early cellular indicator of toxicity where apoptotic induction occurs at lower toxicant concentrations and may switch to necrosis (a pathological condition) at higher concentrations (Robertson and Orrenius, 2000). There are two main reasons why inducible cell suicide exists. First, apoptosis is needed for proper development as is mitosis. For example, in the formation of the fetal fingers and toes, apoptosis is required to remove the tissue between them. Second, apoptosis is needed to destroy cells that pose a threat to the integrity of the organism such as in the cases of viral infection, DNA damage and carcinoma. While the signals triggering apoptosis may vary widely between species, tissues

and conditions, the intracellular apoptotic process is highly evolutionarily conserved (McConkey, 1998). An example of this high conservation includes the specific DNA fragmentation at internucleosomal sites (Gavrieli et al., 1992). Occurrence of apoptosis can be detected by measuring the specific DNA fragmentation in histological sections using terminal dideoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Gavrieli et al., 1992; Tilly and Perez, 1997). Various studies have demonstrated increased cellular apoptosis in fish ovarian follicular cells, skin, medial yolk vein, thymocytes and hepatocytes after exposure to a variety of toxicants (Janz et al., 1997; Marty et al., 1997; Cantrell et al., 1998; Piechotta et al., 1999; Janz et al., 2001; Weber and Janz, 2001).

Heat shock proteins are highly conserved proteins that are synthesized by the cells of all organisms studied so far (Jaattela, 1999; Whitley et al., 1999), including fish (Iwama et al., 1998). There are three major HSP families: HSP90 (85-90 kDa), HSP70 (68-73 kDa), and low molecular weight HSPs (16-47 kDa). During normal unstressed condition, they play crucial roles in protein metabolism by acting as chaperones to assure proper folding and transport of newly formed polypeptides, as well as taking part in the repair and/or degradation of altered or denatured proteins (Kiang and Tsokos, 1998). Heat shock protein induction has been proposed as a biochemical indicator of toxicant exposure in aquatic organisms (Iwama et al., 1998). Among the HSP families, HSP70 subfamily is the best characterized and contains a highly stress-inducible isoform encoded by the HSP70 gene. Varieties of metals (Ryan and Hightower, 1994; Williams et al., 1996; Yoo and Janz, 2003), bleached kraft pulp mill effluents (Janz et al., 1997; Vijayan et al., 1998; Janz et al., 2001), and aryl hydrocarbon receptor (AhR) agonists (Weber and Janz, 2001) have been reported to induce HSP70 expression in fish. Weber and Janz (2001) reported a negative relationship between expression of HSP70 and apoptosis in ovaries of

juvenile channel catfish (*Ictalurus punctatus*) exposed to β-naphthoflavone and dimethylbenz[a]anthracene. Gurbuxani et al. (2003) recently demonstrated that HSP70 can inhibit apoptosis by neutralizing and interacting with apoptosis-inducing factor, providing a plausible explanation for Weber and Janz's (2001) observed negative relationship between HSP70 expression and apoptosis.

Frequently used methods to determine HSP70 expression are via Northern and Western blots, in addition to linking HSP70 gene promoters to reporter genes in transfected cells (Salminen et al., 1996; Braeckman et al., 1999; Ait-Aissa et al., 2000; Tully et al., 2000). More recently, transgenes were developed to provide a simpler and faster technique of detecting HSP70 expression. Transgenes allow for easier observation of gene expression in vivo by fusing a reporter gene to a stress-inducible promoter. Stable germline transgenic zebrafish were recently shown to be possible (Amsterdam et al., 1995; Halloran et al., 2000). Green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria* has been incorporated into zebrafish to produce a line of transgenic zebrafish carrying the HSP70-eGFP construct. This HSP70-eGFP reporter gene was demonstrated to be a reliable and dose-dependent indicator of cadmium exposures (Blechinger et al., 2002). This stable line of transgenic zebrafish has been maintained over many generations and may prove to be a useful tool in determining the mechanism(s) of toxicity associated with xenoestrogen exposures.

1.5 Zebrafish (Danio rerio)

Fish have long been used as sentinels for various types of toxicological studies including risk assessment. For the present study, zebrafish was chosen to evaluate the effects of xenoestrogens, namely EE and NP, on the fish reproductive system. Zebrafish is a small fresh water tropical fish originating from the streams of South-eastern Himalayan region. It is popular among the aquarium hobbyist and readily available in pet stores. In the scientific community, it

has also enjoyed a steady gain in recognition as a choice model for vertebrate developmental and genetic studies. Its value in fish biology, toxicology and pharmaceutical research has also been widely recognized (Lele and Krone 1996). Consequently, an extensive knowledge base of morphological, physiological, biochemical and histological information at all early developmental stages as well as in juveniles and adults of both sexes has been accumulated. Combined with the advancements in mapping of its genome, zebrafish has proven to be an invaluable research species. As a result of its popularity as an animal model, its optimal breeding conditions and maintenance techniques have been determined and publicized (Westerfield, 1995). Its popularity is evident through the dramatic increase in the number of publications using this species; going from about 100 annually in the early 1990s to around 3,500 per year in recent times (Hill et al., 2005).

Zebrafish is an undifferentiated gonochorist, where genotypic males go through a period of transitory hermaphroditism during the juvenile developmental stage (Takahashi, 1977). Other gonochoristic teleosts that undergo a similar process include sea bass (*Dicentrarchus labrax*), European eel (*Anguilla anguilla*), and several other antabantids (Blazquez et al., 1998). All gonads of zebrafish begin with development of ovary-like tissues. Soon afterwards, future females continue to develop ovaries, while in future males, the ovarian tissues degenerate and disappear, followed by the development of testicular tissues at 23-25 days post hatch (dph). Simply put, even the males pass through an ovary-like stage before the final development of male gonads. Full sex reversal and testicular formation occurs by 40 dph (Takahashi, 1977; Uchida et al., 2002). Therefore time of exposure is as critical as type of compounds exposed in determining the effect of xenoestrogens. Several studies have reported that exposing zebrafish to xenoestrogens during the critical period of sexual differentiation results in altered sex

distribution, suppressed gonad development, induction of VTG, and development of ovotestes (Andersen et al., 2003; Hill and Janz, 2003; Orn et al., 2003).

Zebrafish is an attractive alternative animal model for several reasons. First, adult zebrafish are small, hardy and relatively inexpensive to maintain. Its small size also allows for minimal number of slides needed for complete histological evaluation of major organs (Lele and Krone, 1996; Spitsbergen and Kent, 2003). Under optimal conditions, zebrafish can be bred continuously year-round and females lay large quantities of eggs daily. As well, the eggs are transparent and externally fertilized, making easy close observations on the embryo of the different developmental stages taking place; going from a fertilized egg into a larval fish in a relatively short time span of 2 to 4 days. Another benefit of this species is the rapid generation cycle; the time from eggs to mature adults is only 12 -14 weeks, which is advantageous when performing multi-generation reproductive studies. Studies involving chemical exposures during the early developmental stages can be easily performed using the zebrafish as well since the embryos develop outside of the mothers, and chemical exposure assays can be carried out by simply placing the eggs in aqueous solutions containing the chemical (Westerfield, 1995). Furthermore, the European scientific community, the Organization for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency (US EPA) have all recommended the use of zebrafish as a primary model for investigations involving exposure to xenoestrogens and other endocrine disrupting compounds in fishes.

1.6 Research Scope

Hill and Janz (2003) had previously exposed developing zebrafish to differing levels of the weak estrogen receptor agonist, NP (10, 30, or 100 µg/l nominal) or the potent estrogen receptor agonist, EE (1, 10, 100 ng/l nominal) to determine concentration dependent effects on sex distribution, gonad morphology, VTG induction and breeding success. Fish were exposed

from 2 to 60 dph, and after day 60, the surviving fish were reared in clean water until reaching adulthood at 120 dph for breeding studies. The results showed a concentration dependent increase in the number of fish with underdeveloped gonads in EE and NP exposure groups while incidences of ovotestes were associated with NP exposures. Induction of VTG was also evident at 30 and 100 μg/l NP and 10 ng/l EE. Although no significant deviation in sex distribution were observed in adults at 160 dph, significant reductions in the percent of fertilized eggs, hatchability and swim-up success at 10 ng/l EE and 100 μg/l NP were recorded from breeding trials of adult fish from 120 to 160 dph (Hill and Janz, 2003). These results indicate that functional endpoints (breeding success) may be a more sensitive indicator than morphological endpoints (length, weight and condition factor) in adult zebrafish exposed to xenoestrogens during sexual differentiation and early gametogenesis. The study also focused on histological examination of gametogenesis and organ toxicity of the exposed zebrafish. It found that exposure to NP (≥ 100 μg/l nominal) and EE (≥ 1 ng/l nominal) from 2 to 60 dph led to concentration-dependent suppression of gametogenesis in both male and female zebrafish. Severe kidney pathology was also evident in 60dph zebrafish at a threshold of 10 ng/l EE. Fish from the same group that had been reared in clean water from 60 to 300 dph exhibited normal testes and lacked signs of liver or kidney histopathology. However, zebrafish exposed developmentally to 100 μg/l NP exhibited increased ovarian follicle atresia at 300dph (Weber et al., 2003). Atresia is a process where ovarian follicles lose their integrity, degenerate, and are subsequently eliminated prior to ovulation (Byskov, 1978). Apoptosis of granulosa cells that provide hormonal support for the oocyte has been determined to be the primary molecular mechanism for ovarian follicular atresia in mammals and most likely other vertebrates as well (Tilly et al., 1991; Hughes and Gorospe, 1991; Hsueh et al., 1994).

Thus far, the majority of research on effects of xenoestrogens on aquatic species has focused primarily on exposure to single chemicals, despite the fact that they are more likely to be exposed to combinations of such compounds in the environment. Furthermore, studies examining the impacts of xenoestrogens in fish have thus far mostly evaluated morphological changes, but as evidenced by several studies (Bayley et al., 1999; Toft and Baatrup, 2001; Hill and Janz, 2003; Weber et al., 2003) functional changes may be better indicators in studying developmental exposure to estrogens in fish. Although there has been an increased effort to understand mixture toxicity of xenoestrogens (Thorpe et al., 2001; Thorpe et al., 2003; Brian et al., 2005), few studies have investigated *in vivo* effects of xenoestrogen mixtures on early development and subsequent reproduction.

Additionally, despite the recent intense efforts to identify and develop reliable biomarkers to evaluate the effect of xenoestrongens on aquatic organisms, so far, there have only been biomarkers of exposure (VTG and zona radiata induction). Therefore, in order to better understand the toxic mechanism of xenoestrogens on the fish reproductive system, research is needed to identify biomarkers that are capable of relating exposure to impaired reproductive capability. Evaluation of HSP70 expression as well as apoptosis induction may contribute towards the development of reliable biomarkers of effect. As demonstrated by Gurbuxani et al., (2003) as well as in fish by Weber and Janz (2001), HSP70 expression appears to affect the level of apoptotic cell death in the vertebrate gonad.

1.7 Research Objectives

The objectives of the present study were to:

1. Determine the effects of developmental exposure to binary mixtures of EE and NP on VTG induction, HSP70 expression, gametogenesis, sex distribution, and reproductive fitness of zebrafish.

2. Determine the possible mechanism of the observed reproductive failure via correlating HSP70 induction and incidence of gonadal cell (ovary and testis) apoptosis.

1.8 Hypotheses

The formulated hypotheses for the objectives are as follows:

1. The effects of exposure to the binary mixtures will be less than additive. While EE is the more potent estrogen receptor agonist of the two, NP occurs in the environment at concentrations several orders of magnitude higher than EE. The higher concentration of NP may act as functional antagonist to EE by blocking EE's ability to bind to estrogen receptors.
2. The decrease in reproductive function of xenoestrogen - exposed zebrafish is due to degeneration of gonads brought on by apoptosis. Fish which exhibit a decrease in reproductive function after xenoestrogenic exposures would exhibit a higher incidence of apoptosis and lower HSP70 expression in gonadal cells.

CHAPTER 2
MATERIAL AND METHODS

2.1 Test Compounds

17α-Ethinylestradiol (17α-ethinyl-1,3,5[10]-estratriene-3,17β-diol; 98% purity) and 4-nonylphenol (technical grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of EE and NP were prepared in HPLC-grade acetone. Solvent concentration was kept at 0.01% v/v nominal throughout the experiment.

2.2 Experimental Animals

2.2.1 Wild-type Zebrafish

Adult wild-type zebrafish for breeding stock were purchased from Petland, a local pet supply store (Saskatoon, SK, Canada) and housed in a temperature (28 ± 1°C) and photoperiod (16 h light: 8 h dark) controlled environmental chamber at the Toxicology Centre at University of Saskatchewan. Approximately two hundred fish were divided evenly between ten 40 l glass aquaria supplied with dechlorinated tap water (pH: 7.7; conductivity: 380 µS/cm; hardness: 128 mg/l $CaCO_3$; alkalinity: 78 mg/l $CaCO_3$; dissolved organic carbon: 2.5 mg/l; total dissolved solid: 210.8 mg/l). Aeration and filtration were provided by Biofoam sponge filters (Hagen, Montreal, QC, Canada). Fish were fed twice per day with Nutrafin Max flake food (Hagen, Montreal, QC, Canada) in the morning, supplemented with freshly hatched brine shrimp (*Artemia nauplii*) in the evening. Fish were acclimated to laboratory conditions for 4 weeks prior to breeding.

2.2.2 HSP70-eGFP Transgenic Zebrafish

Twelve adult HSP70-eGFP transgenic zebrafish were generously donated by Dr. Patrick Krone of the Department of Anatomy and Cell Biology at University of Saskatchewan. They were housed in one 40 l glass aquarium under the same maintenance conditions prescribed for the wild-type zebrafish.

2.3 Parental Breeding

Wild-type adults were bred to obtain eggs for the exposure experiments. Plastic spawning trays covered by meshed lid (with artificial spawning plants attached) were placed in tanks in the afternoon of the day prior to breeding. On the following day, at 1 h after the start of the light cycle, spawning trays were removed from the tanks. Eggs were collected and placed into plastic Petri dishes containing sterile salt-based egg water (60 mg/l Instant Ocean salts) (Westerfield, 1995) and assessed for viability under a dissecting microscope. Fertilized eggs are translucent while non- fertilized ones appear opaque. Fertilized eggs were then divided into 25ml sterile glass Petri dishes with approximately 50 eggs per dish and rinsed using egg water to remove any waste matter. The egg water was changed daily to prevent growth of mold or bacteria. Fertilized eggs were held in sterile glass Petri dishes until hatch. Petri dishes used for the incubation of eggs were washed and rinsed with acetone, followed by a thorough rinse with ddH_20. The Petri dishes were then wrapped in aluminum foil and autoclaved at 110°C for 15 min. They were stored in the temperature controlled environmental chamber until use.

Adult HSP70-eGFP transgenic zebrafish were bred to obtain eggs using the same method as the wild-type zebrafish described above.

2.4 Juvenile Zebrafish Care

Prior to starting the 60dph exposure experiment, efforts were devoted to creating a maintenance routine that ensured optimum survival rate of the juvenile zebrafish, particularly

during the critical 7-10 day period following the onset of exogenous feeding. See Appendix A for a description of the various conditions and feeding regimens employed for these experiments.

2.5 Exposure Assays

Newly hatched fry were held in sterile glass Petri dishes (50 fry per dish) containing egg water. At 2 days post-hatch (dph), fry were exposed to nominal concentrations EE (1 or 10 ng/l; denoted EE1 or EE10), NP (10 or 100 µg/l; denoted NP10 or NP100), EE + NP (1 ng/l + 10 µg/l, 1 ng/l + 100 µg/l, 10 ng/l + 10 µg/l, or 10 ng/l + 100 µg/l; denoted EE1+NP10, EE1+NP100, EE10+NP10, or EE10+NP100) or acetone solvent (control) at a 1 µl/10 ml total dilution in system water (Figure 2.1). There were three replicates of each test chemical concentration and solvent control. A 100% water change was performed every 48 h from 2 to 60 dph. Fresh acetone or test chemicals were added at the time of each water change. Two sets of glassware were assigned to each treatment group, and were used to house only the assigned group to prevent any cross-contamination. From 2 to 30 dph, fry were fed an alternating diet of *Paramecium multimicronucleatum* or freshly hatched brine shrimp three times daily. If present, any debris, waste matter or dead fry were removed manually via a Pasteur pipette. At 7 dph, fry were transferred to aerated 250 ml beakers. At 30 dph, fry were transferred to aerated 1 l glass beakers, and an alternating diet of flake food and newly hatched brine shrimp was provided twice daily. Chemical exposures continued until 60 dph. At 60 dph, 42 fish (14 fish per replicate) from each treatment group were randomly selected for measurement of lengths, weights, histological examination of the gonads, HSP70, VTG and apoptosis determinations. Remaining fish were transferred to 20 l glass aquaria and reared in dechlorinated municipal tap water to allow a six month depuration period until 240 dph, at which time breeding trials were conducted (Figure 2.2).

The initial culture of *Paramecium multimicronucleatum* was supplied by The Bug Farm (San Rafael, CA, USA). The culture was maintained by adding the starter culture to a 1 l glass beaker containing 15-20 boiled wheat kernels in 900 ml dechlorinated water. Every two weeks, a new culture was started by adding paramecium from the previously established cultures to another 1 l glass beaker containing boiled wheat berries in 900 ml dechlorinated water. Cultures were kept in the environmental chamber where warm temperature (28 ± 1 ºC) and light exposure ensured optimum growth.

Brine shrimp (*Artemia nauplii*) were hatched daily in the environmental chamber. In the afternoon prior to the collection of freshly hatched brine shrimp, 1 teaspoon of brine shrimp eggs (San Francisco Bay Brand, Newark, CA, USA) were added to 1 l of water containing 30 g Instant Ocean salts in a separatory funnel. The water was constantly agitated and aerated via an air stone attached to an aquarium air pump. The eggs were allowed to hatch overnight. On the following day, the aeration was turned off and the freshly hatched brine shrimp would collect at the bottom of the beaker due to their weight, while empty egg shells floated to the top. Since brine shrimp are phototropic, a table lamp was directed towards the bottom of the beaker to attract them. Subsequently, brine shrimp were collected and fed to the fish.

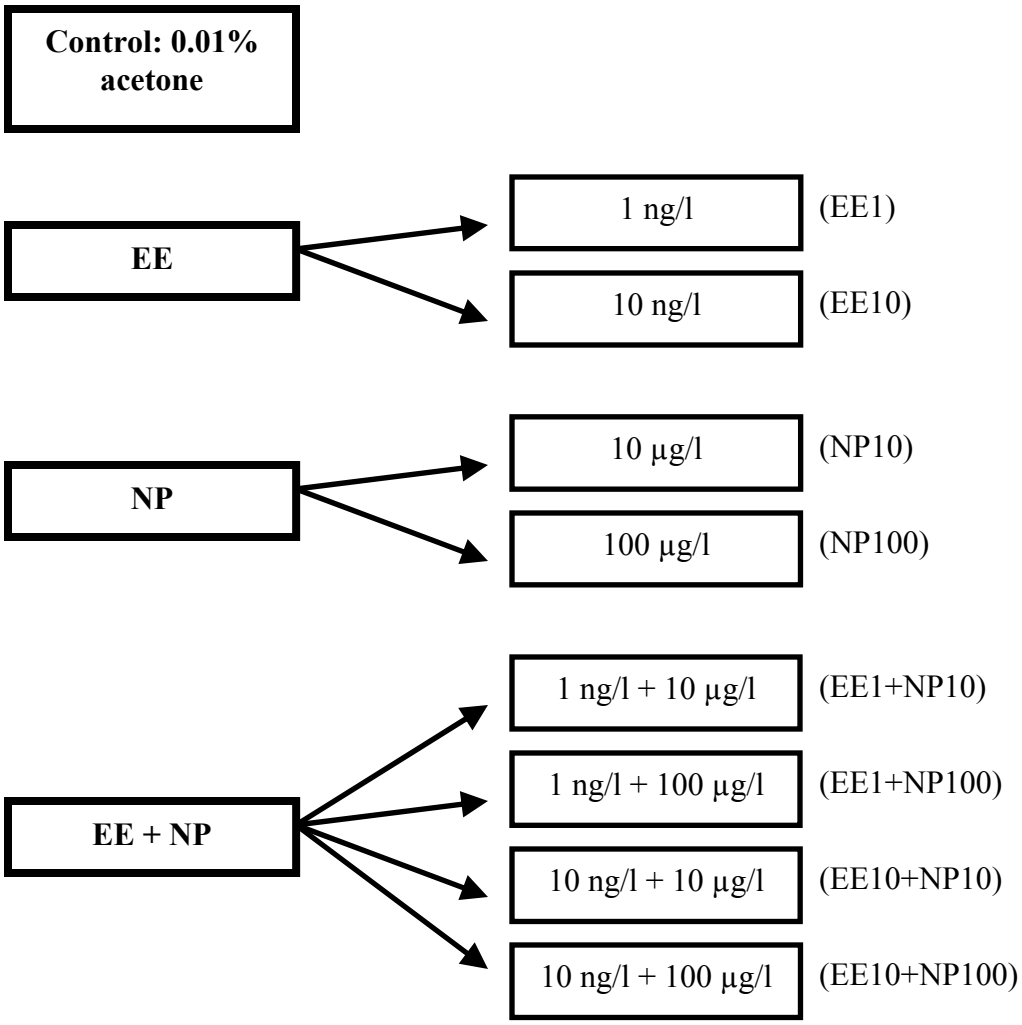


Figure 2.1 Exposure schematic. Three replicates (50 fish per replicate) of each treatment were carried out from 2 to 60 dph. EE, ethinylestradiol; NP, nonylphenol.

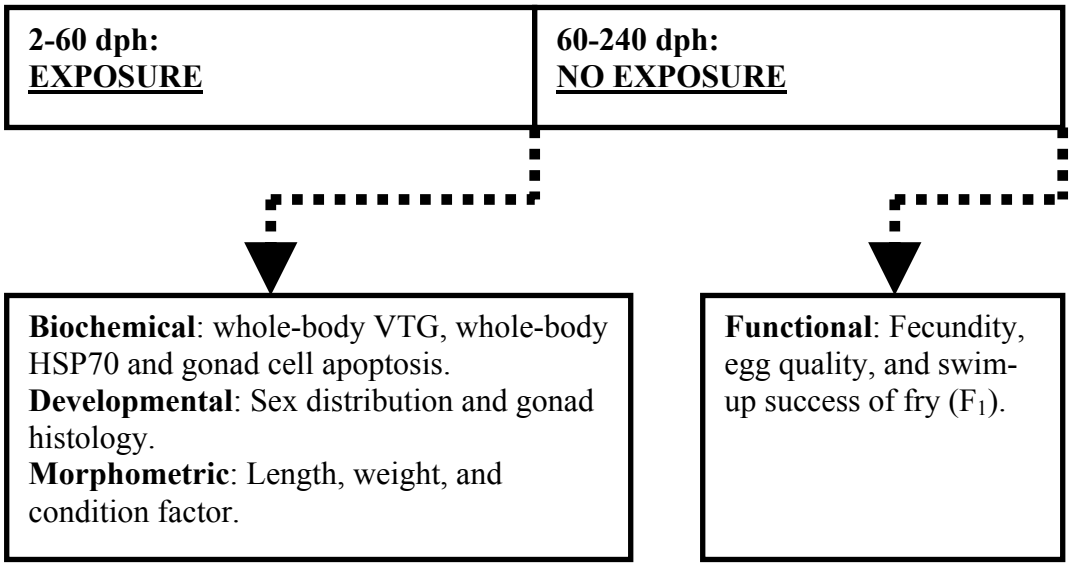


Figure 2.2 Experimental design. Exposure to EE, NP, EE+NP or control from 2 to 60 dph. At 60 dph, 42 fish (14 fish per replicate) from each treatment group were randomly selected for measurement of biochemical endpoints (whole-body VTG induction, whole-body HSP70 expression, gonadal apoptosis), developmental endpoints (sex distribution and gonad histology), and morphometric endpoints (lengths, weights, and condition factor) Remaining fish were reared in dechlorinated municipal tap water to allow a six month depuration period until 240 dph for reproductive studies to evaluate functional endpoints (fecundity, egg quality, and swim-up success of the F_1 generation). EE, ethinylestradiol; NP, nonylphenol; VTG, vitellogenin.

2.6 Histology

At 60 dph, 21 fish from each treatment were euthanized with an overdose of MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt). Body weights and lengths were recorded, and condition factors were calculated using the following formula (Ricker, 1975):

$$[(\text{body weight (g)}/\text{length (mm)}^3] \times 100{,}000 \quad (2.1)$$

Fish were then fixed in Bouin's solution (Ricca Chemical, Arlington, TX, USA) for 24 h and subsequently transferred to 70% ethanol. Whole fish were dehydrated through a graded series of ethanol (three consecutive cycles of 70% ethanol for 15 min, 2 h and 2h, followed by 2 h in 80% ethanol, 2 h in 95% ethanol, three consecutive 2 h-cycles in absolute ethanol, then two consecutive 40 min-cycles in xylene) and embedded in paraffin wax using Fisher Histomatic

Tissue Processor model 165 (Fisher Scientific, Hampton, NH, USA). The paraffin-embedded specimens were sent to Prairie Diagnostics at the University of Saskatchewan (Saskatoon, SK, Canada) for sectioning. Longitudinal 5 μm sections along the entire dorso-ventral axis were taken with a microtome at 20 μm increments, and collected onto glass slides.

2.6.1 Hematoxylin and Eosin Staining

The slide-mounted sections were deparaffinized and stained with hematoxylin and eosin with the following protocol. Deparaffinization was achieved by soaking the sections in xylene twice at 2 min each. The sections were then hydrated by soaking in absolute ethanol for 2 min, a 1-sec dip in another absolute ethanol bath, a 1-sec dip in 95% ethanol, and a 1-sec dip in 70% ethanol. The staining process begun by first soaking the sections in hematoxylin for 5 min, followed by 3 1-sec dips in tap water, a quick dip in acid alcohol, immersed in warm tap water for 6 min, 4 quick dips in ddH_2O and concluded with soaking in eosin for 3 min. Sections were rinsed with tap water to remove any extra stain. Sections were then dehydrated by a 1-sec dip in 70% ethanol, a 1-sec dip in 95% ethanol, a 1-sec dip in absolute ethanol and a 2-min soaking in another absolute ethanol bath. Two additional soaks in xylene for 2 and 5 min were performed before the cover slips were mounted on the sections with Permount (Fisher Scientific, Hampton, NH, USA). The slides were allowed to dry overnight under a fume hood. Slides were then analyzed blind of treatment with a light microscope to evaluate the presence or absence of gonad tissue and determine the phenotypic sex.

2.6.2 Terminal Dideoxynucleotidyl Transferase dUTP Nick End Labeling

Prior to starting this assay, the incubator, oven, tubes and benchtop used were thoroughly disinfected with ethanol. As well, only sterilized pipette tips were used.

Additional sections from the previously-sectioned paraffin-embedded specimens were taken with a microtome, and collected onto glass slides for TUNEL staining. The slides were

deparaffinized by incubating at 60°C in a drying oven for 30 min. The slides were hydrated by first soaking in xylene (replaced everyday) for two 5-min sessions. Following the xylene soaks, the slides were soaked in 100% isopropanol for 5 min. This step was repeated for an addition 5 min. The slides were subsequently soaked in 90% isopropanol for 3 min, 80% isopropanol for 3 min, 70% isopropanol for 3 min and concluding with a 3-min soak in sterile reagent-grade water (replaced everyday).

The slides were placed in a Shandon immunohistochemistry tray, topped with a bead of citrate digestion buffer (0.1 M trisodium citrate), and covered with Shandon cover slips while ensuring no air bubbles were present. Each slide was rinsed with 100 µl citrate digestion buffer. This was repeated two more times. The slides were then placed in a microwave and heated for 30 sec. Immediately after, slides were rinsed with 100 µl ice-cold ddH_2O. This was repeated two additional times. For the positive control slide, 100 µl DNase working solution was added and incubated at room temperature for 10 min. One ml of DNase working solution was made up of 980 µl of DNase buffer (30 mM Tris base, 140 mM potassium cacodylate, 4 mM $MgCl_2 \cdot 6H_2O$, pH 7.2), 10 µl of 10 mM dithiothreitol and 10 µl of DNase I (Roche 10776785001). The positive control slide was washed three times with 200 µl sterile water (5 min between each wash). All slides were pre-incubated with 95 µl 1x TdT substrate solution for 20 min at room temperature. The 1x TdT substrate solution was made from the following recipe: 25 mM Tris HCl, 200 mM cacodylic acid, 5 mM cobalt chloride, pH 6.6. On the day of assay, 1 µl per ml of TdT substrate solution of dUTP-fluorescein (Roche 11373242910) was added. The mixture was protected from light with aluminum wrap and stored on ice. The negative control slide was treated with 100 µl of 1x TdT substrate solution while the other slides were each treated with 100 µl of TUNEL reaction mixture (995 µl 1x TdT substrate solution and 5 µl TdT

enzyme (Roche 03333566001) to make 1 ml). All slides were placed in a humidified incubator at 37°C for 120 min. Afterwards, the slides were rinsed three times with 100 µl TBS at room temperature; 5 min in between each rinse. The slides were then incubated with 100 µl per slide of 3% BSA in TBS for 30 min at room temperature. Ninety-five µl of Converter-AP solution (Roche 11684809910) was added to each slide and incubated at 37°C for 60 min. Subsequently, each slide was rinsed three times with 100 µl TBS at room temperature; 5 min in between each rinse. The slides were each incubated with 100 µl NBT/BCIP working solution for 60 min at room temperature. During the 60-min incubation, three additional treatments of 100 µl NBT/BCIP working solution were added to each slide at 15-min intervals. The NBT/BCIP working solution was made by diluting the NBT/BCIP stock solution (Roche 11681451001) 1:50 with dilution buffer (0.1 M Tris base, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5). Due to the light sensitive nature of the color development process, lights were turned off and the slides covered with aluminum foil. The color development was stopped by rinsing slides with 200 µl TE at room temperature. This was repeated two additional times with 1 min in between each rinse.

The slide-mounted sections were dehydrated and counter-stained by first dipping in eosin working solution (1% (w/v) eosin Y solid, 78% (v/v) 95% ethanol, 0.5% glacial acetic acid) for 10 sec. The slides then were immersed in 70% isopropanol for 30 sec, 90% isopropanol for 5 min, 100% isopropanol for 3 min (twice) and xylene for 2 min (twice). A drop of Permount was added to each slide and mounted with a cover slip. The slides were allowed to dry overnight under a fume hood. The slides were examined using an Olympus AH-2 light microscope.

2.7 Gametogenesis

Staging of oogenesis and spermatogenesis in hematoxylin and eosin-stained histological sections was conducted following the quantitative method described by Weber et al. (2003). Staging of the ovarian development was performed on sections of histologically determined

female fish. With 200 × magnification using an Olympus AH-2 light microscope, the number of ovarian follicles at oogonial (smallest in size, with relatively larger nucleus contained in eosinophilic ooplasm) and previtellogenic (small in size, basophilic ooplasm and large nucleus with visible chromatin and single somatic cell layer) stages were enumerated. The percent of follicles at each developmental stage was calculated as a percent of the total number of follicles in each view. Four replicate views were evaluated in a blind fashion for each fish.

Staging of the testicular development was performed on sections of histologically determined male fish. Using an Olympus AH-2 light microscope at 1000 × magnification, the number of spermatocysts containing spermatogonia (eosinophilic cytoplasm with relatively large nucleus), primary or secondary spermatocytes (thread-like or condensed chromatin, respectively, with relatively smaller cytoplasm that does not take up dye) and spermatids or mature sperm (tightly packed nuclear material lacking surrounding cytoplasm and with a developed tail) stages were enumerated. The percent of spermatocysts at a given developmental stage was calculated as a percent of the total number of spermatocysts in each view. Four replicate views were evaluated in a blind fashion for each fish.

2.8 Sixty Days Post-hatch Whole Body Vitellogenin Determination

The remaining 60 dph fish were stored at -80°C until determinations of VTG and HSP70. Vitellogenin levels were determined using a commercial ELISA kit for zebrafish VTG (Biosense Laboratories SA, Bergen, Norway) in 60 dph zebrafish whole body homogenates.

Sixty dph zebrafish were homogenized 1:3 (w:v) with ice cold working PBS buffer. Working PBS buffer was made by first diluting 10X PBS buffer (10.9 g Na_2HPO_4, 3.2 g NaH_2PO_4, 90 g NaCl, and 1 l ddH_2O) 1:10 with ddH_2O to create 100 ml 1X PBS buffer. The 1X PBS buffer was then formulated to contain 1% BSA and 1mM 2-aminoethyl-benzenesulfonyl

fluoride (AEBSF). The homogenates were centrifuged for 15 min at 3,000g and 4°C. Following centrifugation, the supernatant was withdrawn and stored at -80 °C.

As per manufacturer's instruction, duplicate 100 µl of each homogenate sample (diluted appropriately to yield readings in the detection range of the VTG standard curve; dilution factor ranged from 1:20 to 1:1,000,000) was pipetted into the wells of the 96-well ELISA plate pre-coated with a capture antibody that specifically binds to VTG. Eight serial concentrations of the VTG standard were added in duplicate to each plate. The plate was incubated for 60 mins at room temperature. Each well was then washed 3 times with 200 µl washing buffer (PBS, 0.05% Tween-20). Subsequently, 100 µl diluted detecting antibody was added to each well and incubated for 60 mins at room temperature. The plate was washed 3 times with 200 µl washing buffer per well. To each well, 200 µl diluted secondary antibody was added and incubated for 60 mins at room temperature. Each well was then washed 5 times with 200 µl washing buffer, followed by the addition of 100 µl OPD-peroxidase substrate solution. The plate was incubated in the dark, covered with aluminum foil, for 30 minutes at room temperature. The color development was stopped with the addition of 50 µl 2M H_2SO_4 to each well. The absorbance was subsequently read at 492 nm with a microplate reader. Vitellogenin concentrations measured were normalized to the weight (g) of the corresponding sample, taking into account the dilution of the sample. Results were expressed as mg VTG per g body weight.

2.9 Sixty Days Post-hatch Whole Body HSP70 Determination

Whole body homogenates of 60 dph zebrafish were prepared to determine HSP70 expression using Western blotting. Individual fish were homogenized in 200 µl ice cold buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% v/v Triton X-100, 10% v/v glycerol, pH 7.5) containing protease inhibitors (0.1 mg/ml AEBSF, 20 µg/ml soybean trypsin inhibitor, and 1.9 µg/ml aprotinin). Samples were gently mixed for 1 h at 4°C, and then

centrifuged for 25 min at 5,200 rpm and 4°C. Following centrifugation, the supernatant was withdrawn and stored at -80 °C. The protein concentration of each sample was determined (DC Protein Assay, BioRad, Hercules, CA, USA) using bovine serum albumin as standard.

Proteins (30 µg protein per lane) were separated using 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The solution for the separating gel was made from the following ingredients: 16.0 ml lower stock solution (1.5 M Tris HCl, 0.4% SDS to pH 8.8), 21.36 acrylamide/Bis stock (30%/0.8% w/v), 26.36 ml ddH_2O, 320 µl 10% ammonium persulfate and 28 µl TEMED. The solution was vortex mixed prior to and after the addition of TEMED. The solution was subsequently poured into the casting case and the separating gel was cast. One ml of butanol was layered to the top of the separating gel solution; this allows removal of any air bubbles at the top of the gel and ensures this part of the gel does not dry out. Upon polymerization of the separating gel after 30 min, the butanol was poured out of the casting case, and the gel was rinsed with ddH_2O. At this point, the solution for the stacking gel was prepared using the following items: 5.0 ml upper stock (0.5 M Tris HCl, 0.4% SDS to pH 6.8), 3.0 ml acrylamide/Bis stock (30%/0.8% w/v), 12.0 ml ddH_2O, 60 µl 10% ammonium persulfate and 20 µl TEMED. The stacking gel solution was vortex mixed prior to and after the addition of TEMED. A 15-well comb was inserted into the casting case prior to the addition of the stacking gel solution. Air bubbles were removed by gently tapping and/or reinserting the comb. To ensure the wells were of the proper depth, additional stacking gel solution was periodically added as the stacking gel begun to polymerize. Upon complete polymerization of the stacking gel, the homogenized samples were diluted 1:1 with 2x SDS sample buffer (0.05 M Tris-base, 1% SDS, 0.01% bromophenol blue, and ddH_2O to 100.0 ml). Immediately before use, β-mercaptoethanol was added to the SDS sample buffer at a concentration of 20 µl per 1.0 ml

buffer. Prior to loading into the stacking gel, the diluted samples were heated at 80°C for 10 min with a heating block. In lane #1, 6 µl of kaleidoscope marker (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to provide a molecular weight standard. Samples were loaded into the other lanes using gel-loading pipette tips. Additional 2x SDS sample buffer was added to ensure equal volume in each well. The electrophoresis chamber was filled with running buffer (247.8 mM Tris base, 1.918 M glycine, 1% SDS and ddH_2O to 4 l, then diluted 1:10 with ddH_2O) and proteins were separated at 64 mA for 4 hours at room temperature. Following separation, proteins were transferred to 0.45 µm nitrocellulose membranes (BioRad) while immersed in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, 0.1% SDS and ddH_2O to 5 l) for 17 hours at 4°C, at 30 V.

Membranes were then blocked for 1 h in a 5% skim milk (BioRad) solution in TBS-T. Membranes were probed with a 1:5,000 dilution of a monoclonal mouse anti-bovine HSP70 antibody (Sigma-Aldrich H5147) in 1% skim milk/TBS-T for 1 h at room temperature. The membranes were then washed three times (5, 10 and then 15 min) with 100 ml TBS-T at room temperature. Membranes were then incubated at room temperature with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% skim milk/TBS-T solution for 1 h at room temperature. The membranes were then washed three times (5, 10 and then 15 min) with 100 ml TBS-T at room temperature. Membranes were visualized using NBT/BCIP color development. Immediately before use, 300 µl of NBT stock (0.1 g NBT, 1.4 ml DMSO and 0.6 ml ddH_2O) and 165 µl of BCIP stock solution (0.1 g BCIP and 2 ml ddH_2O) were added to 50 ml of NBT/BCIP reaction buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2 \cdot 6H_2O$ to 1 l, pH 9.5). The membranes were then incubated with the NBT/BCIP developing solution in a tinfoil-covered

tray on a shaker for 70 min. The color development reaction was stopped upon the addition of 0.2 M EDTA (pH 7.0). HSP70 immunoreactive bands were quantitated by densitometry using an Epson 4180 scanner and Scion image beta (version 4.02) software.

2.10 Reproductive Studies at 240 Days Post-hatch

Upon completion of the exposure study at 60 dph, the remaining fish were reared in dechlorinated municipal tap water and allowed a 6 month depuration period, at which time the reproductive study commenced. Three replicates of 15 breeding trials were conducted. For each replicate, 12 randomly selected fish from each treatment were placed into a breeding tank. Each trial consisted of placing spawning trays into the breeding tanks to induce spawning behavior, followed by egg collection the next day. Fish were rested 2 days (absence of spawning trays in the tank) between trials. Assessed parameters included total cumulative egg production, total number of eggs per breeding trial, percent viability (percentage of total eggs that were fertilized), percent hatchability (percentage of total fertilized eggs that hatched), and percent swim-up success (as percentage of fertilized eggs). Following the breeding trials, fish from each treatment were euthanized, weights and lengths were recorded, and individual fish were sexed under a dissecting microscope. The gonads of each fish were removed and weighed to determine the gonadosomatic index (GSI), calculated as:

$$\text{(weight of the gonads in mg) / (weight of the total body in mg)} \times 100 \qquad (2.3)$$

Ovaries and testes dissected from adult fish were used to determine gonadal HSP70 expression as described for 60 dph fish.

2.11 Statistical Analysis

All data were tested for normality using the Kolmogorov-Smirnov test. If assumptions of normality and equal variance held true, then two-way analysis of variance (ANOVA) was performed to assess if any interaction exists between EE and NP on the examined endpoints.

The different levels of EE were categorized into EE0, EE1, and EE10 while levels of NP were categorized into NP0, NP10, and NP100, creating a 3x3 two-away ANOVA matrix (Table 2.1). If an interaction was detected, further one-way ANOVA followed by Tukey's post-hoc test was conducted to assess the significance of these effects. If assumptions of normality and equal variance failed, non-parametric Kruskal-Wallis one-way ANOVA on Ranks test was used followed by Multiple Comparison versus the respective control groups (Dunn's method). Chi-square analysis was used to identify differences in sex distribution between control and each treatment group. Statistical significance was set at $\alpha = 0.05$.

Table 2.1 3x3 Two-way ANOVA matrix for evaluating the interaction effects between EE and NP on the examined endpoints. EE, ethinylestradiol; NP, nonylphenol.

	EE0	**EE1**	**EE10**
NP0	EE0NP0 (Control)	EE1NP0 (EE1)	EE10NP0 (EE10)
NP10	EE0NP10 (NP10)	EE1NP10 (EE1+NP10)	EE10NP10 (EE10 + NP10)
NP100	EE0NP100 (NP100)	EE1NP100 (EE1 + NP100)	EE10NP100 (EE10 + NP100)

CHAPTER 3
RESULTS

3.1 Sixty Days Post-hatch Survival

Mean survival rates from 2-60 dph were 77.3 ± 3.7% (CON); 77.3 ± 4.1% (NP10); 61.3 ± 12.9% (NP100); 83.3 ± 5.7% (EE1); 78.7 ± 5.7 (EE10); 61.3 ± 10.7 (EE1+NP10); 74.0 ± 2.3% (EE1+NP100); 74.7 ± 9.3% (EE10+NP10) and 88.0 ± 3.1% (EE10+NP100). There were no significant differences in the 60 dph survival rate among treatments (Fig. 3.1).

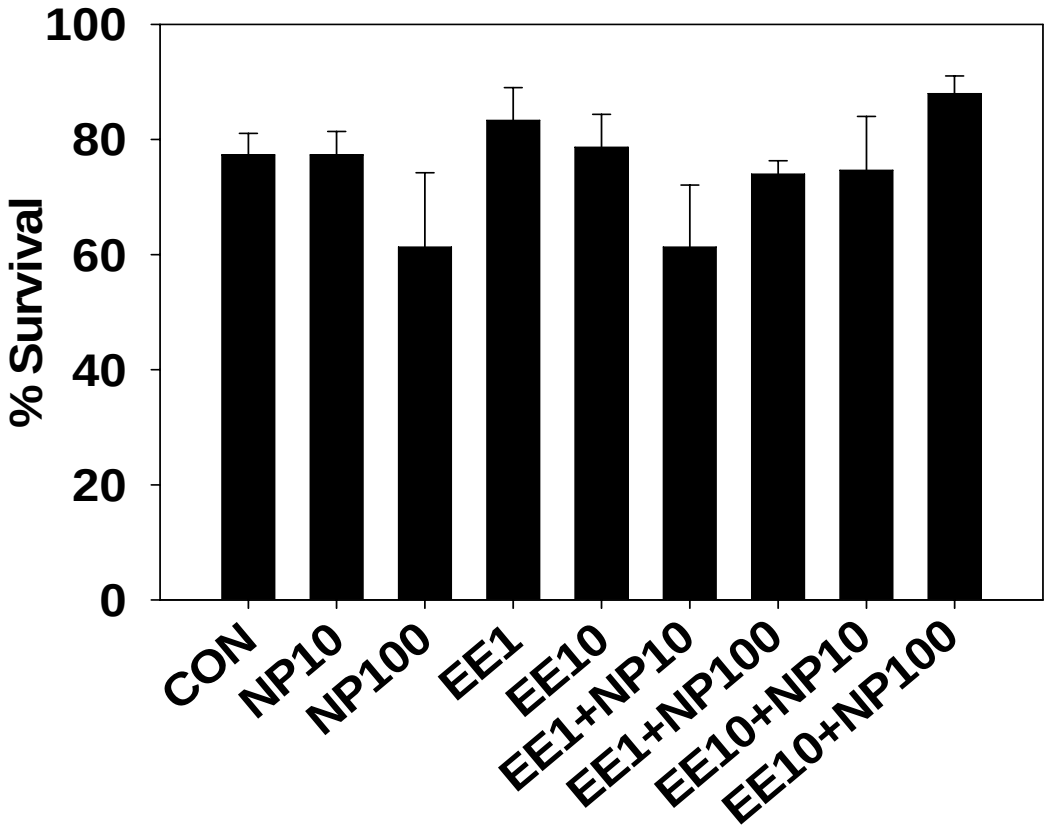


Figure 3.1 Percent survival of 60 days post-hatch zebrafish. Percent survival observed at 60 days post-hatch in zebrafish exposed to 10 or 100 µg/l nonylphenol (NP10 or NP100 respectively), 1 or 10 ng/l 17α-ethinylestradiol (EE1 or EE10 respectively), 1ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE1+NP10), 1ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE1+NP100), 10ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE10+NP10), 10ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE10+NP100) or solvent control (CON, 0.01% acetone v/v) from 2 to 60 days post-hatch. Initial clutch size was 50 fish per replicate, with three replicates per treatment. Data are means ± SEM, one-way ANOVA followed by Tukey's test.

3.2 Sixty Days Post-hatch Sex Distribution and Gonad Histology

3.2.1 Control Zebrafish

Histological evaluation of gonads from whole mount sections of control zebrafish at 60 dph showed a sex distribution of 29.4% female and 53.0% male (Fig. 3.2) with respective normal ovarian and testicular ultrastructure and progression of gametogenesis. In the ovaries, a greater

part of the gonad tissue was made up of previtellogenic oocytes (81.7 ± 2.3%; Fig. 3.3), surrounded by oogonia and early oocyte stages (18.3 ± 2.3%) in the caudal and cranial peripheries (Fig. 3.4b). In histologically determined males, 88.9% showed a full spectrum of sperm cell differentiation stages (Fig. 3.4a). Gonad staging was conducted on all histologically determined males. However, due to the lack of males in the majority of the treatment groups, statistical analysis was not performed. In addition to the identified male and female, 17.6% of the control fish were classified as having undifferentiated gonads, containing primordial germ cells with no discernable cells characteristic of either sex (Fig. 3.4c).

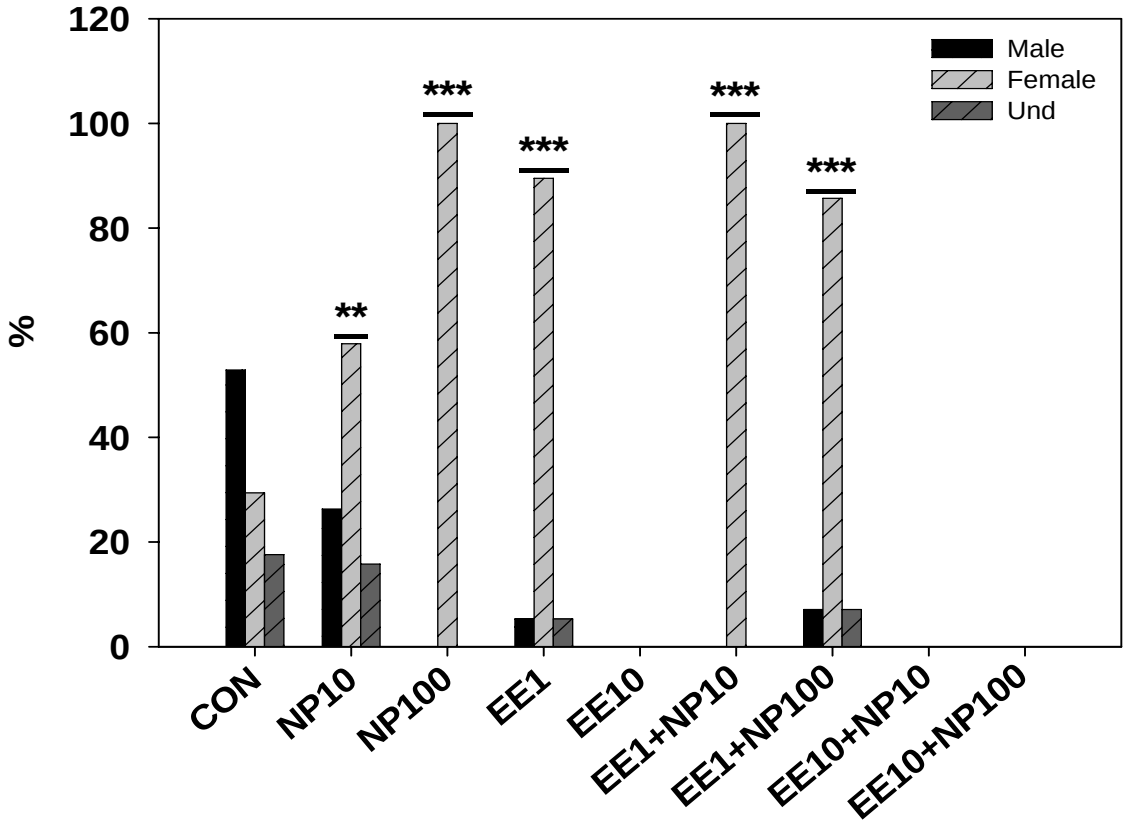


Figure 3.2 Sex distribution of 60 days post-hatch zebrafish. Sixty days post-hatch zebrafish were exposed to 10 µg/l nonylphenol (NP10; $n = 19$), 100 µg/l nonylphenol (NP100; $n = 16$), 1 ng/l 17α-ethinylestradiol (EE1; $n = 19$), 1 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE1+NP10; $n = 14$), 1 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE1+NP100; $n = 14$), or solvent control (CON, 0.01% acetone v/v; $n = 17$) from 2 to 60 days post-hatch. No discernable gonadal tissues were observed in groups exposed to 10 ng/l 17α-ethinylestradiol (EE10), 10 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE10+NP10), or 10 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE10+NP100). Significantly different from control using chi-square test: ** $P < 0.001$; *** $P < 0.0005$. Und: undifferentiated gonad.

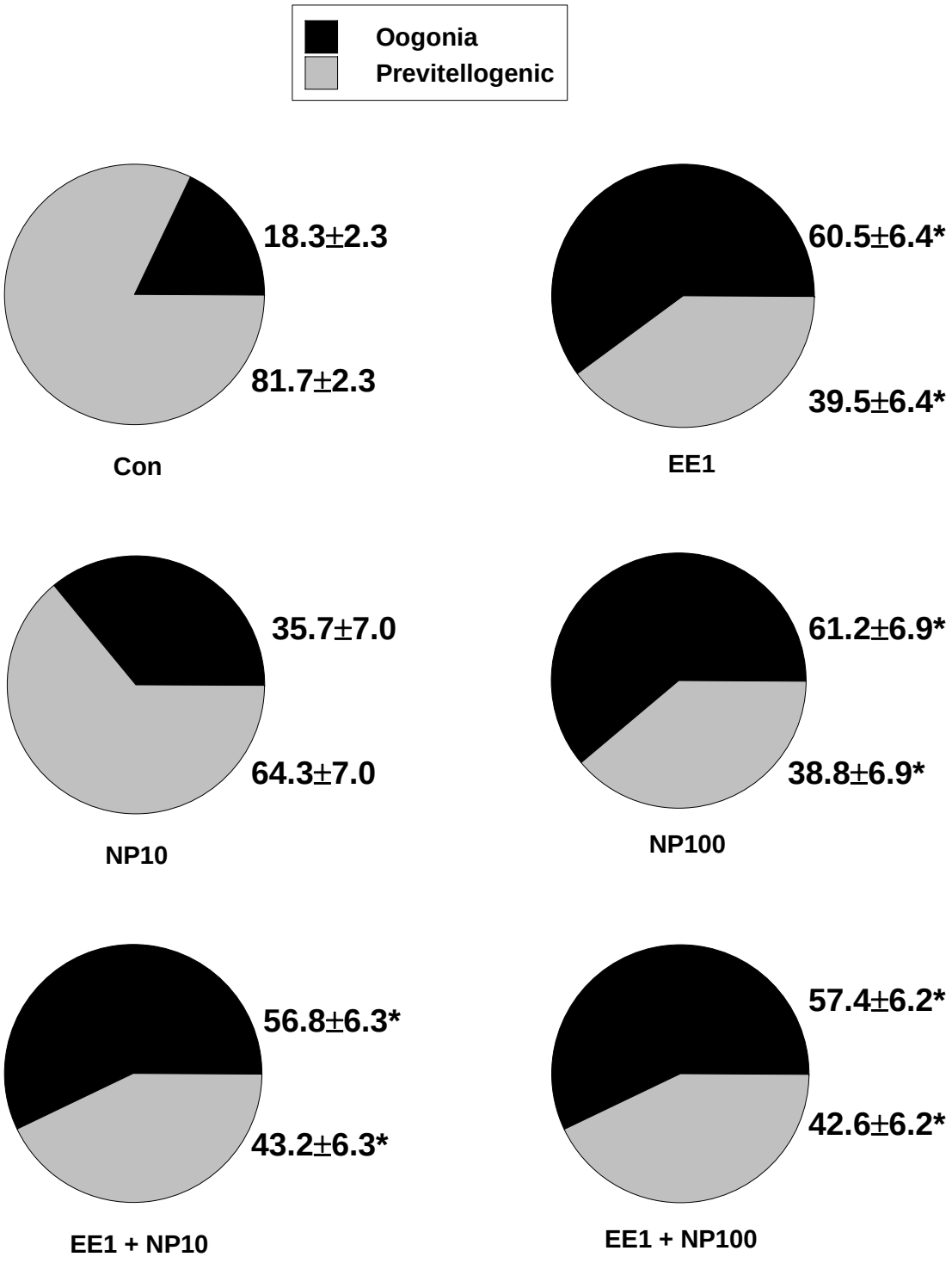
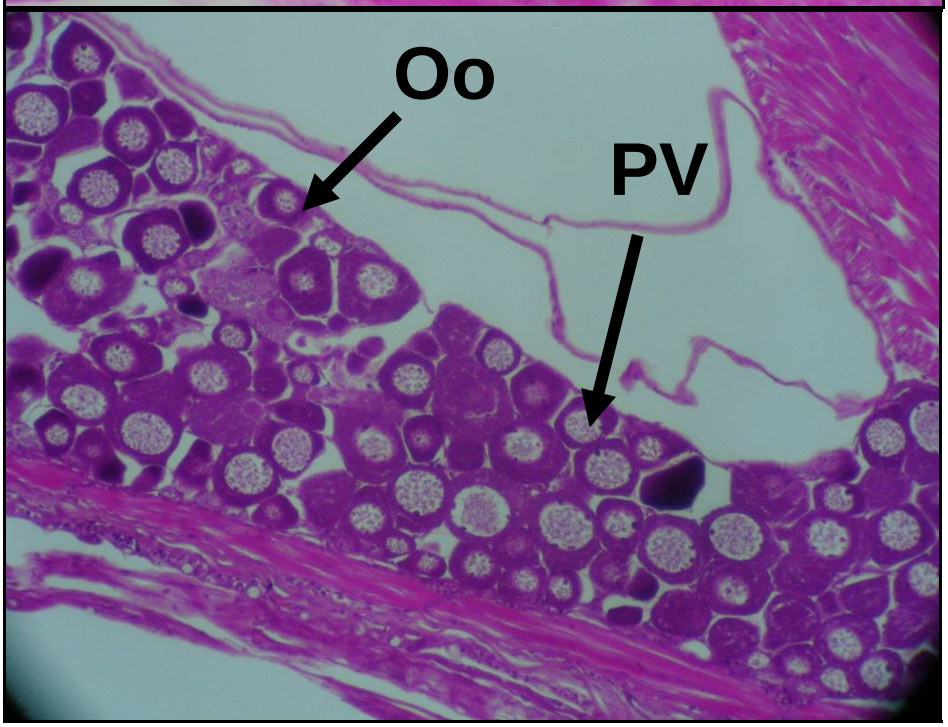

Oogonia
Previtellogenic
18.3±2.3
81.7±2.3
Con
60.5±6.4*
39.5±6.4*
EE1
35.7±7.0
64.3±7.0
NP10
61.2±6.9*
38.8±6.9*
NP100
56.8±6.3*
43.2±6.3*
EE1 + NP10
57.4±6.2*
42.6±6.2*
EE1 + NP100

Figure 3.3 Ovarian oogenesis staging of 60 days post-hatch female zebrafish. Percent distribution of different stages of ovarian follicle development in zebrafish exposed from 2 to 60 days post-hatch to 10 or 100 μg/l nonylphenol (NP10; $n = 11$ or NP100; $n = 16$ respectively), 1 ng/l 17α-ethinylestradiol (EE1; $n = 17$), 1 ng/l 17α-ethinylestradiol + 10 μg/l nonylphenol (EE1 + NP10; $n = 14$), 1 ng/l 17α-ethinylestradiol + 100 μg/l nonylphenol (EE1 + NP100; $n = 12$) or solvent control (CON; $n = 5$). Four replicate views were evaluated blinded at 200 × magnification in each fish and results were calculated using the mean value from each (n) fish. Numerical values (mean ± S.E.M) for the percent of total at each stage of ovarian follicle development observed are indicated adjacent to the corresponding pie area. Significantly different from solvent control using one-way ANOVA followed by Tukey's test: * $P<0.05$.

(a)

(b)



(c)

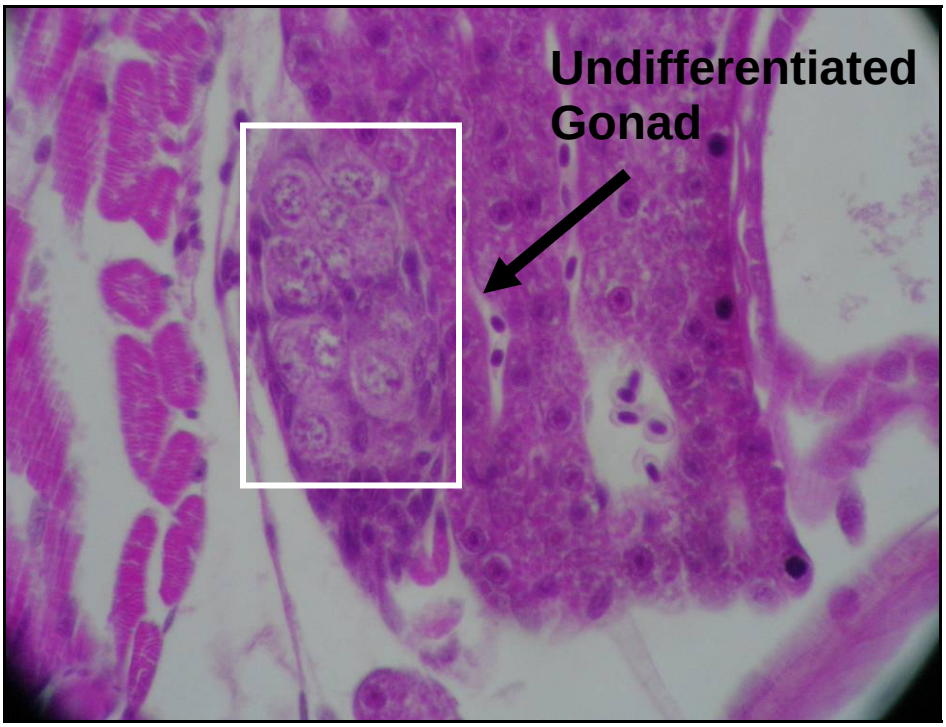


Figure 3.4 Representative hematoxylin and eosin-stained gonad sections of 60 days post-hatch zebrafish. (a) Testis of control male at 800 × magnification, (b) ovary of control female at 320 × magnification, (c) undifferentiated gonad (inside white box) of a fish exposed to 1 ng/l 17α-ethinylestradiol from 2 to 60 days post-hatch at 800 × magnification. Sc: Spermatocyte; Sg: spermatogonia; M: mature sperm; PV: previtellogenic follicle; Oo: oogonia.

3.2.2 Nonylphenol-only Exposure

Fish exposed to NP10 had 57.9% female, 26.3% male, and 15.8% undifferentiated, which was significantly different from the control group ($P < 0.001$; Fig. 3.2). In females, the ovaries contained mainly previtellogenic oocytes (64.3 ± 7.0%) with the rest being oogonia and early oocyte stages (35.7 ± 7.0%; Fig. 3.3). Among the histologically determined males exposed to NP10, differences in the level of testicular development was observed among fish, with approximately 60% showing the full array of sperm cell differentiation and 40% exhibiting only the early stages of differentiation. Fish exposed to NP100 were 100% female, with the ovaries consisting of 38.8 ± 6.9% previtellogenic oocytes and 61.2 ± 6.9% oogonia (Fig. 3.2). The sex

distribution and ovarian gametogenesis of the NP100 group were significantly different from the control ($P < 0.0005$ and $P < 0.05$, respectively). No evidence of ovotestes was present in either of the two NP-only treatments.

3.2.3 Ethinylestradiol-only Exposure

The sex distribution of EE1-exposed fish was significantly different when compared with the control ($P < 0.0005$), with the majority being female (89.4%), while 5.3% male and 5.3% undifferentiated made up the remaining population (Fig. 3.2). The ovaries were comprised mainly of oogonia (60.5 ± 6.4%) with 39.5 ± 6.4% previtellogenic oocytes, which was significantly different than the control ($P < 0.05$; Fig. 3.3). The single fish identified histologically as male had testes displaying only the early stages of sperm cell differentiation. There were no discernable gonadal tissues present in fish exposed to 10 ng/l of EE (Fig. 3.2). No evidence of ovotestes was present in either of the two EE-only treatments.

3.2.4 Binary Mixture Exposure

Fish exposed to EE1+NP10 resulted in 100% female (Fig. 3.2), whose ovaries contained a roughly equal portion of oogonia and previtellogenic oocytes (56.8 ± 6.3% and 43.2 ± 6.3% respectively; Fig 3.3). In the EE1+NP10 exposure group, sex distribution and ovarian gametogenesis were significantly different from the control ($P < 0.0005$ and $P < 0.05$, respectively). Exposure to EE1+NP100 produced 85.8% female, 7.1% male and 7.1% undifferentiated; this distribution was significantly different when compared with the control ($P < 0.0005$). The ovaries in this group contained 57.4 ± 6.2% oogonia and 42.6 ± 6.2% previtellogenic oocytes, which was significantly different than the control ($P < 0.05$). The one individual identified as having testes showed the full range of sperm cell differentiation stages. There were no discernable gonadal tissues present in fish exposed to treatment groups containing

10 ng/l of EE (EE10, EE10+NP10 and EE10+NP100; Fig 3.2). No evidence of ovotestes was present in any of the binary treatments.

3.3 Sixty Days Post-hatch Length, Weight, and Condition Factor

At 60 dph, only fish in treatments containing 10 ng/l of EE (EE10, EE10+NP10 and EE10+NP100), the same groups that showed no discernable gonadal tissues, exhibited decreased length and weight, but higher condition factor, compared with control fish (Table 3.1; $P < 0.001$ for all except condition factor of EE10+NP100, where $P < 0.01$) . The remaining treatments were not significantly different in length, weight, or condition factor compared with the control (Table 3.1).

Table 3.1 Length, weight, and condition factor of 60 days post-hatch zebrafish. Length, weight, and condition factor determined in 60 days post-hatch zebrafish exposed to 10 or 100 µg/l nonylphenol (NP10 or NP100 respectively), 1 or 10 ng/l 17α-ethinylestradiol (EE1 or EE10), 1 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE1+NP10), 1 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE1+NP100), 10 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE10+NP10), or 10 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE10+NP100), or solvent control (0.01% acetone v/v) from 2 to 60 days post-hatch.

Treatment	Length (mm)	Weight (mg)	Condition factor
Control ($n = 42$)	12.5 ± 0.3	13.5 ± 0.9	0.66 ± 0.01
NP10 ($n = 42$)	13.2 ± 0.3	15.6 ± 0.9	0.64 ± 0.01
NP100 ($n = 42$)	13.0 ± 0.4	16.5 ± 1.5	0.78 ± 0.14
EE1 ($n = 42$)	12.7 ± 0.2	13.4 ± 0.7	0.66 ± 0.04
EE10 ($n = 42$)	9.1 ± 0.2***	6.3 ± 0.3***	0.84 ± 0.03***
EE1+NP10 ($n = 42$)	13.3 ± 0.4	17.3 ± 1.3	0.69 ± 0.01
EE1+NP100 ($n = 42$)	12.3 ± 0.4	13.5 ± 1.1	0.68 ± 0.01
EE10+NP10 ($n = 42$)	9.5 ± 0.2***	7.8 ± 0.6***	0.84 ± 0.03***
EE10+NP100 ($n = 42$)	10.0 ± 0.2***	7.8 ± 0.4***	0.76 ± 0.02**

Data are mean ± SEM. Significantly different from control using Dunn's multiple comparisons: **$P < 0.01$ and ***$P < 0.001$.

3.4 Sixty Days Post-hatch Vitellogenin Induction

At 60 dph, the gender of fish used for VTG analyses was not determined due to the species' lack of prominent sexual dimorphism at this developmental stage. Therefore, the VTG concentrations presented here are mean values of combined genetic male and female fish. Two-way ANOVA indicated that both NP and EE alone had a significant effect on 60 dph VTG induction ($P < 0.0005$ and < 0.0001 respectively). Furthermore, there was a significant interaction between EE and NP on VTG induction ($P < 0.0001$). Subsequently, one-way ANOVAs were conducted on each subgroup to examine the effect of different levels of NP (NP0, NP10 or NP100) at each level of EE (EE0, EE1 or EE10) on VTG induction at 60 dph (Fig. 3.5).

In the absence of EE (EE0), whole body VTG levels in the NP100-exposed, but not NP10-exposed, zebrafish were significantly greater than the control group ($P < 0.05$; Fig. 3.5). In the presence of 1ng/l EE, the addition of 10 µg/l NP (EE1+NP10) increased the VTG significantly ($P < 0.05$) when compared to EE1 alone. However, with the addition of 100 µg/l NP, the VTG level in EE1+NP100 was not significantly different from EE1 (Fig. 3.5). Analysis from the EE10-exposed groups showed that VTG induction in EE10+NP100 was significantly lower than EE10+NP10 ($P < 0.05$) as well as EE10 ($P < 0.001$) exposure groups (Fig. 3.5). One-way ANOVA comparing EE-only exposure groups with the control indicated that VTG induction in EE1 was not significantly different than the control group, while EE10 had a significantly higher VTG induction than the control group ($P < 0.001$).

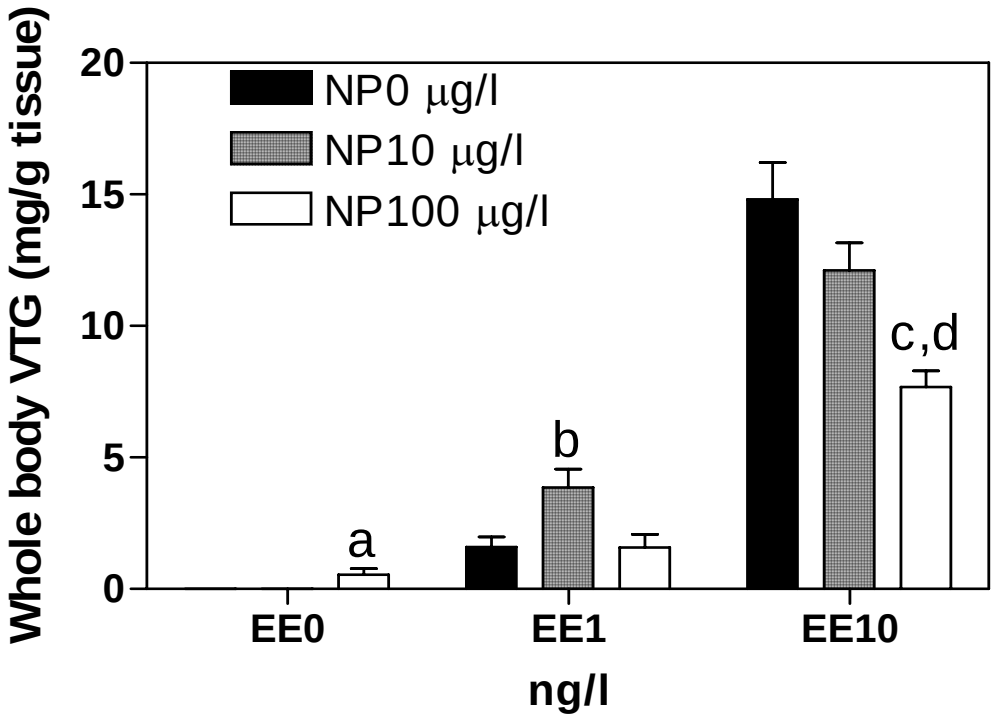


Figure 3.5 Whole body vitellogenin concentration of 60 days post-hatch zebrafish. Sixty days post-hatch (dph) zebrafish were exposed to 10 or 100 μg/l nonylphenol (NP10 or NP100 respectively; $n = 10$), 1 or 10 ng/l 17α-ethinylestradiol (EE1 or EE10 respectively; $n = 10$), 1 ng/l 17α-ethinylestradiol + 10 μg/l nonylphenol (EE1+NP10; $n = 9$), 1 ng/l 17α-ethinylestradiol + 100 μg/l nonylphenol (EE1+NP100; $n = 10$), 10 ng/l 17α-ethinylestradiol + 10 μg/l nonylphenol (EE10+NP10; $n = 11$), or 10 ng/l 17α-ethinylestradiol + 100 μg/l nonylphenol (EE10+NP100; $n = 10$), or solvent control (CON, 0.01% acetone v/v; $n = 10$) from 2 to 60 days post-hatch. Data are mean ± SEM. Two-way ANOVA indicates a significant interaction between EE and NP on 60 dph VTG ($P < 0.001$). Subsequent one-way ANOVA of each subgroup, followed by Tukey's test: (a) Significant difference between NP100 and control ($P < 0.05$); (b) significant difference between EE1+NP10 and EE1 ($P < 0.001$); (c) significant difference between EE10+NP100 and EE10+NP10 ($P < 0.05$); (d) significant difference between EE10+NP100 and EE10 ($P < 0.001$).

3.5 Sixty Days Post-hatch Whole Body HSP70 Induction

Two-way ANOVA indicated that individual treatments of EE or NP had no significant effect on 60 dph whole body HSP70 expression. As well, there was no interaction detected between EE and NP on HSP70 expression (Fig. 3.6).

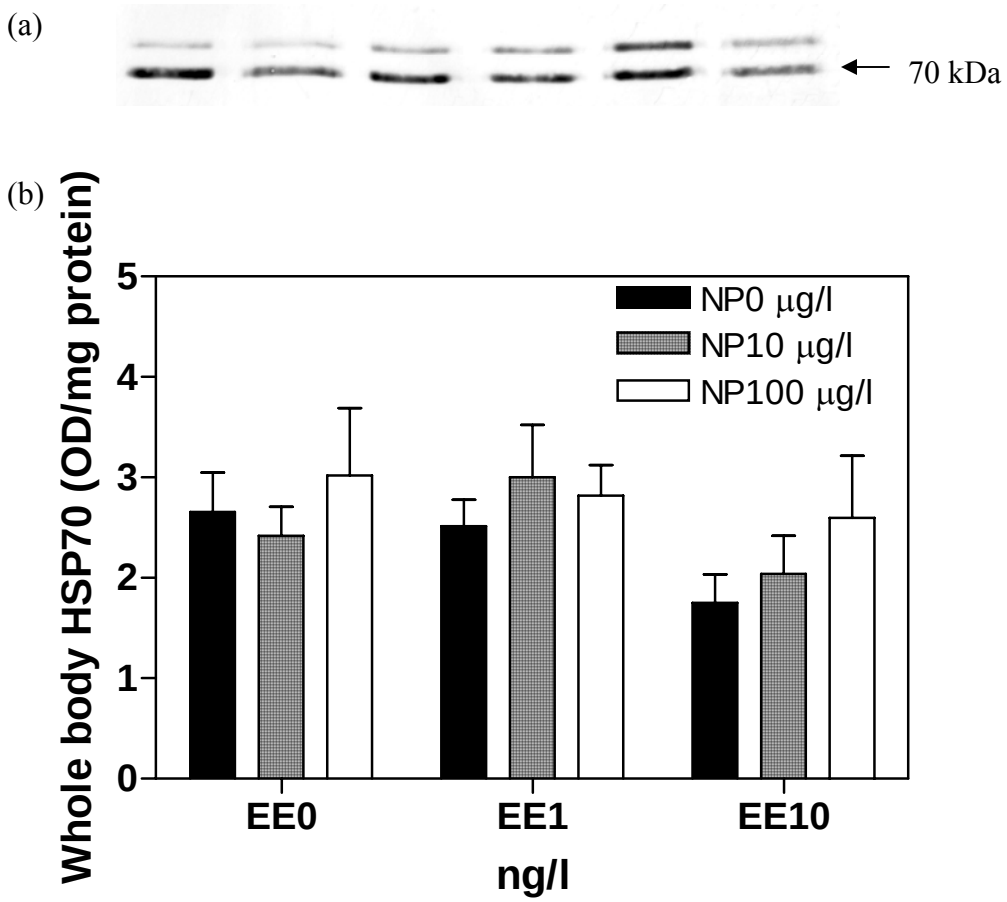


Figure 3.6 Whole body heat shock protein 70 expression of 60 days post-hatch zebrafish. Whole body heat shock protein 70 expression determined using Western blotting of 60 days post-hatch zebrafish exposed to 10 or 100 μg/l nonylphenol (NP10 or NP100 respectively), 1 or 10 ng/l 17α-ethinylestradiol (EE1 or EE10 respectively), 1 ng/l 17α-ethinylestradiol + 10 μg/l nonylphenol (EE1+NP10), 1 ng/l 17α-ethinylestradiol + 100 μg/l nonylphenol (EE1+NP100), 10 ng/l 17α-ethinylestradiol + 10 μg/l nonylphenol (EE10+NP10), or 10 ng/l 17α-ethinylestradiol + 100 μg/l nonylphenol (EE10+NP100), or solvent control (CON, 0.01% acetone v/v) from 2 to 60 days post-hatch. (a) Representative immunoblot. Lane 1: NP10; lane 2: NP100; lane 3: CON; lane 4: EE1+NP10; lane 5: EE1+NP100; lane 6: EE10+NP10 (b) Densitometry of immunoreactive bands. Data are mean optical density (OD) ± S.E.M, one-way ANOVA followed by Tukey's test.

3.6 Sixty Days Post-hatch Gonadal Apoptosis

Unfortunately, excessive amounts of tissue were sectioned from the preserved samples for the 60 dph sexing and gonadal staging, leaving very little to no gonadal tissues in the

remaining samples. Of the slides that were TUNEL stained, none contained gonadal tissues upon microscopic examination.

3.7 Adult Breeding Experiments

After a recovery period of 6 months in clean water, zebrafish were assessed for their reproductive fitness at 240 dph. A successful breeding event was defined as the production of one or more eggs. The highest percentage of successful trials was in the control group with 95.6% (43/45; 43 successful breeding events out of 45 conducted, Fig. 3.7a), and the lowest percentage of successful trials was observed in the EE10+NP10 group with 0% (0/45). The percentages of successful trials in other groups were as follows: NP10 (88.9%; 40/45), NP100 (88.9%; 40/45), EE1 (71.1%; 32/45), EE10 (40.0%; 18/45), EE1+NP10 (86.7%; 39/45), EE1+NP100 (86.7%; 39/45), and EE10+NP100 (57.8%; 26/45). From the 45 breeding trials conducted in each treatment, it appeared that fish from groups exposed to 10 ng/l EE, alone or in combination, were the most adversely affected with respect to cumulative egg production. Zebrafish exposed to EE10, EE10+NP10, and EE10+NP100 had the lowest cumulative egg production, with 1965, 0, and 1808 eggs, respectively (Figure 3.7b). Control, NP10, and EE1+NP100 groups had the highest cumulative egg production, with 10,752, 11,275, and 12,754 eggs, respectively. Data from the remaining groups include EE1 with 4926 eggs, NP100 with 6705 eggs, and EE1+NP10 with 8545 eggs (Fig. 3.7b).

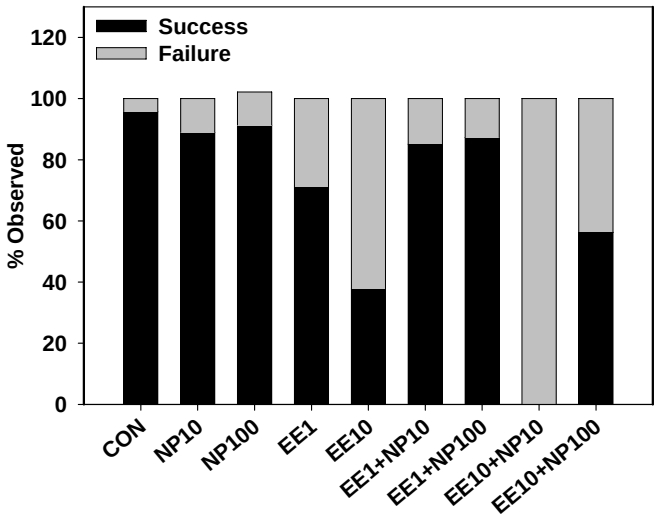
Two-way ANOVA of the mean number of eggs produced per breeding trial indicated that EE-only treatments significantly decreased egg production ($P < 0.0001$). Although treatments of NP alone did not significantly affect the egg production, a significant interaction between NP and EE on egg production was detected ($P < 0.0001$). Subsequent one-way ANOVA conducted on the EE0 subgroups indicated that neither NP10 nor NP100 had a significant impact on egg production (Fig. 3.7c). In 1 ng/l EE-exposed fish, the addition of 10 µg/l NP (EE1+NP10) did

not significantly influence egg production when compared with EE1, however the addition of 100 µg/l NP (EE1+NP100) increased egg production significantly when compared with EE1 ($P < 0.01$; Fig. 3.7c). With the EE10-containing groups, egg production from EE10+NP10 was significantly decreased when compared with EE10 and EE10+NP100 ($P < 0.01$ for both), while no significant difference was detected between EE10 and EE10+NP100. One-way ANOVA comparing the control group with EE-only groups indicated that both EE1 and EE10 had significantly reduced mean egg production ($P < 0.001$ for both; Fig. 3.7c).

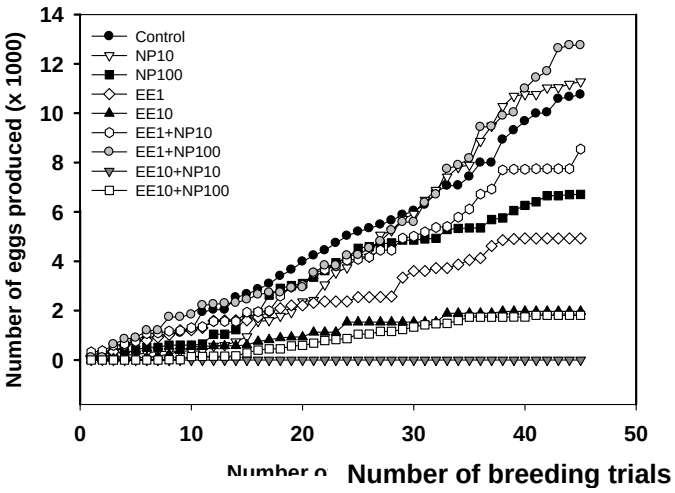
One of the criteria for two-way ANOVA is that the sample size of each of the examined populations had to be greater than 1. Since zebrafish exposed to EE10+NP10 did not produce any eggs during the 45 breeding trials conducted, there were no subsequent data on egg viability and hatchability, as well as swim-up success of the F1 generation. Consequently, two-way ANOVA was not performed on these endpoints. However, with one-way ANOVA, it was demonstrated that fish previously exposed to 10 µg/l of NP did not exhibit any significant differences in viability (78.7 ± 2.0%), hatchability (90.6 ± 1.9%) or F1 swim-up success (80.6 ± 2.2%) when compared with the control (80.1 ± 2.4% viability, 84.2 ± 3.0% hatchability, and 89.9 ± 1.2% swim-up success; Fig. 3.7d). Fish exposed to EE1 had a significant decrease in egg viability (63.6 ± 4.2%, $P < 0.01$) but no differences in hatchability (85.5 ± 3.6%) or swim-up success (81.8 ± 3.6%) when compared with the control. Fish in the NP100 and the EE1+NP10 exposure groups showed significant reductions in F1 swim-up success (74.7 ± 2.8% and 73.2 ± 2.5% respectively, $P < 0.001$) but no differences in viability (75.5 ± 2.8% and 71.6 ± 3.2% respectively) and hatchability (78.0 ± 2.7% and 88.6 ± 2.4% respectively) of eggs as compared to the control. Groups EE1+NP100 and EE10+NP100 produced fish that showed significant reductions in egg viability (63.2 ± 3.8% and 62.6 ± 3.7% respectively, $P < 0.001$) and F1 swim-

up success (71.6 ± 3.4% and 69.9 ± 4.5% respectively, $P < 0.001$) but not hatchability (85.6 ± 2.8% and 84.9 ± 3.1% respectively) compared with the control. Fish exposed to EE10 showed significant reductions in egg viability (51.4 ± 7.1%, $P < 0.001$), hatchability (64.4 ± 7.1%, $P < 0.01$) and F1 swim-up success (59.1 ± 4.0%, $P < 0.001$) when compared with the control.

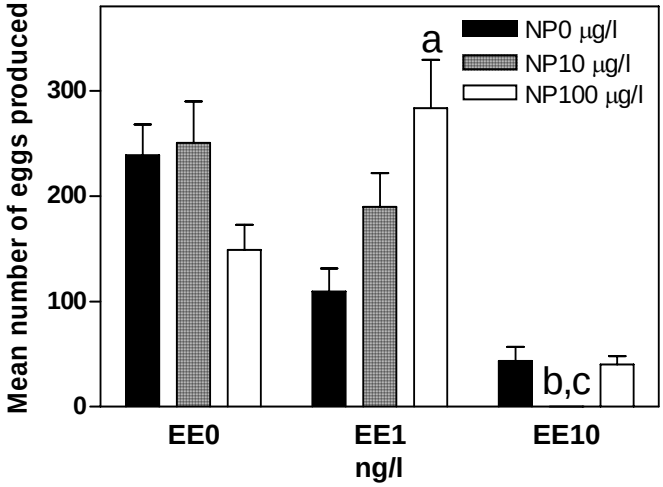
(a)


Success
Failure
% Observed
120
100
80
60
40
20
0
CON
NP10
NP100
EE1
EE10
EE1+NP10
EE1+NP100
EE10+NP10
EE10+NP100

(b)


Control
NP10
NP100
EE1
EE10
EE1+NP10
EE1+NP100
EE10+NP10
EE10+NP100
Number of eggs produced (x 1000)
14
12
10
8
6
4
2
0
0
10
20
30
40
50
Number of breeding trials

(c)


NP0 μg/l
NP10 μg/l
NP100 μg/l
Mean number of eggs produced
300
200
100
0
a
b,c
EE0
EE1
EE10
ng/l

(d)

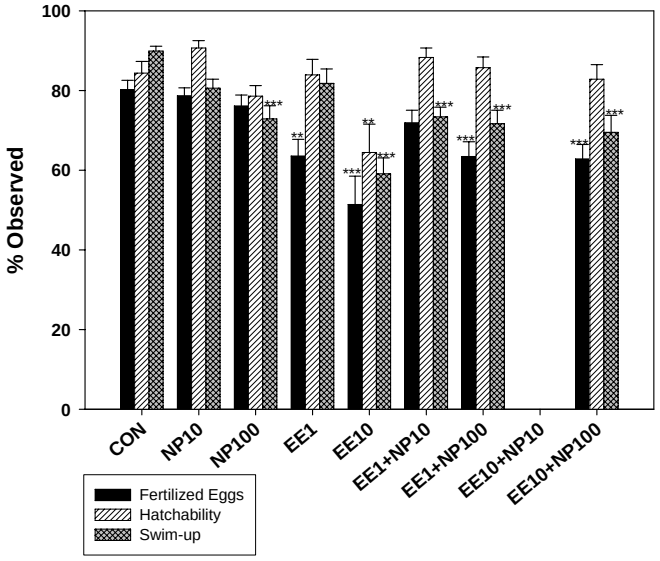

% Observed
100
80
60
40
20
0
CON
NP10
NP100
EE1
EE10
EE1+NP10
EE1+NP100
EE10+NP10
EE10+NP100
Fertilized Eggs
Hatchability
Swim-up

Figure 3.7 (a) Percent of successful breeding trials, (b) total cumulative number of eggs spawned, (c) mean number of eggs produced per breeding trial, and (d) percent fertilized eggs (fertilized eggs), percent hatch (hatchability), and percent F_1 swim-up success (swim-up) determined using adult (240 dph) zebrafish previously exposed to 10 or 100 µg/l nonylphenol (NP10 or NP100 respectively), 1 or 10 ng/l 17α-ethinylestradiol (EE1 or EE10), 1 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE1+NP10), 1 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE1+NP100), 10 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE10+NP10), or 10 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE10+NP100), or solvent control (CON, 0.01% acetone v/v) from 2 to 60 days post-hatch. Two-way ANOVA indicated a significant interaction between EE and NP on mean number of eggs produced ($P < 0.0001$). Subsequent one-way ANOVA, followed by Tukey's test, indicated: (a) significant difference between EE1+NP100 and EE1 ($P < 0.01$); (b) significant difference between EE10+NP10 and EE10 ($P < 0.01$); (c) significant difference between EE10+NP10 and EE10+NP100 ($P < 0.01$). Significantly different from solvent control using one-way ANOVA followed by Tukey's test: ** $P < 0.01$; *** $P < 0.001$.

3.8 Adult Sex Distribution

At the end of the reproductive trials, the gender of fish from all treatments were determined under a dissecting microscope. The control group had 58.7% males and 41.3% females (Fig. 3.8). The NP10 exposure group contained 53.8% males and 46.2% females, while NP100 contained 75.8% males and 24.2% females, which was statistically significant when compared with the control ($P < 0.05$). The EE1 exposure group consisted of 61.2% males and 38.8% females, whereas the EE10 exposure group had a predominantly male population with 92.2% males and 7.8% females, which was significantly different from the control ($P < 0.0005$). The EE1+NP10 group was comprised of 70.0% males and 30.0% females, and EE1+NP100 contained 64.3% males and 35.7% females. Neither of these groups had a significantly different sex distribution when compared with the control group. Both of the EE10-containing mixture exposure groups had a primarily male population with EE10+NP10 having 98.0% males and 2.0% females (significantly different from the control, $P < 0.0005$), and EE10+NP100 consisting of 93.1% males and 6.9% females (significantly different from the control, $P < 0.0005$).

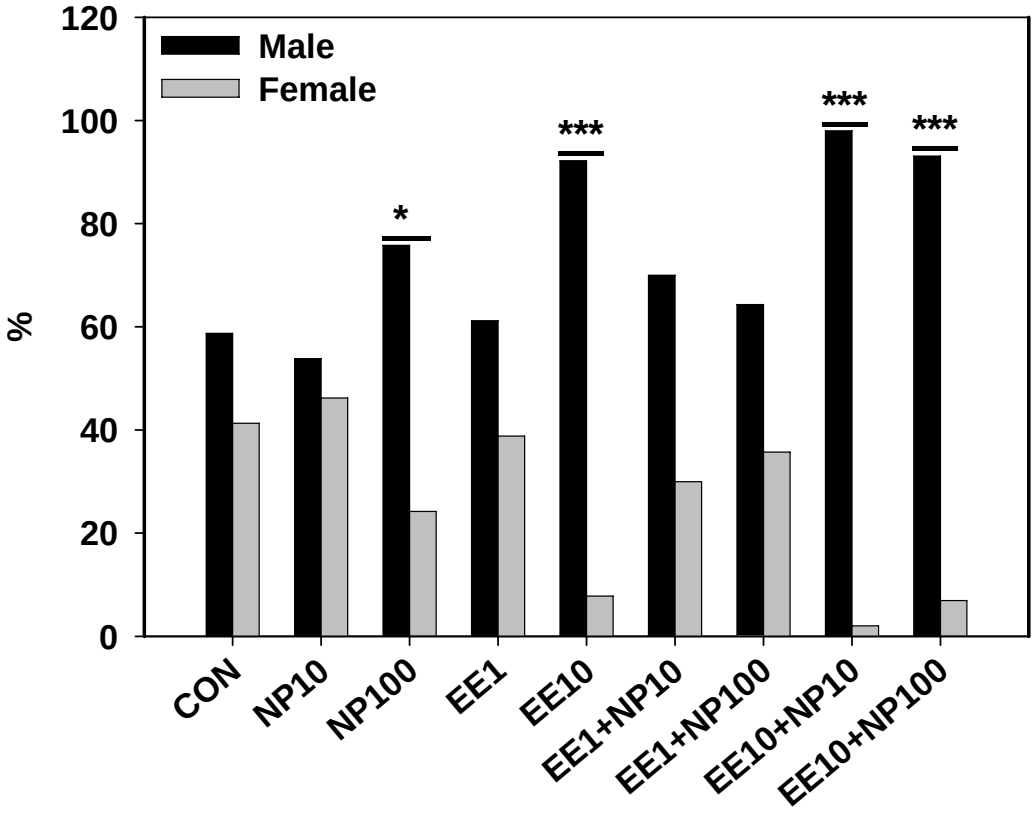


Figure 3.8 Sex distribution of adult zebrafish. Sex distribution of adult zebrafish previously exposed to 10 or 100 µg/l nonylphenol (NP10; *n* = 52 or NP100; *n* = 33 respectively), 1 or 10 ng/l 17α-ethinylestradiol (EE1; *n* = 64 or EE10; *n* = 63 respectively), 1 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE1+NP10; *n* = 40), 1 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE1+NP100; n = *42*), 10 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE10+NP10; *n* = 49), or 10 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE10+NP100; *n* = 72), or solvent control (CON, 0.01% acetone v/v; *n* = 63) from 2 to 60 days post-hatch. Significantly different from control group using chi-square test: * $P < 0.05$; *** $P < 0.0005$.

3.9 Adult Length, Weight, Condition Factor and Gonadosomatic Index

Fish in the highest binary exposure group (EE10+NP100) had significantly lower weights ($P < 0.05$) and shorter lengths ($P < 0.05$), but no difference in condition factor when compared with the control (Table 3.2a). Fish in the NP100 exposure group were not significantly different in lengths or weights, but had a significantly higher condition factor when compared with the control ($P < 0.05$). The remaining treatments exhibited no significant differences in length,

weight, or condition factor. With the exception of significantly lower GSI in EE10 adult males, no differences in GSI from any treatments were observed when compared with the control (Table 3.2b).

Table 3.2 (a) Length, weight, and condition factor and (b) gonadosomatic index (GSI) determined in adult zebrafish previously exposed to 10 or 100 µg/l nonylphenol (NP10 or NP100 respectively), 1 or 10 ng/l 17α-ethinylestradiol (EE1 or EE10), 1 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE1+NP10), 1 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE1+NP100), 10 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE10+NP10), or 10 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE10+NP100), or solvent control (0.01% acetone v/v) from 2 to 60 days post-hatch.

Treatment	Weight (mg)	Length (mm)	Condition factor
(a)			
Control (*n* = 63)	616 ± 34	39.6 ± 0.5	0.94 ± 0.01
NP10 (*n* = 52)	596 ± 39	39.2 ± 0.6	0.93 ± 0.02
NP100 (*n* = 33)	631 ± 29	39.3 ± 0.4	1.02 ± 0.02*
EE1 (*n* = 67)	580 ± 31	39.1 ± 0.4	0.93 ± 0.02
EE10 (*n* = 65)	535 ± 43	38.1 ± 0.6	0.87 ± 0.02
EE1+NP10 (*n* = 39)	649 ± 33	40.1 ± 0.4	0.98 ± 0.02
EE1+NP100 (*n* = 39)	634 ± 28	39.5 ± 0.3	1.01 ±0.02
EE10+NP10 (*n* = 49)	580 ± 22	39.9 ± 0.3	0.91 ± 0.02
EE10+NP100 (*n* = 72)	484 ± 24*	37.6 ± 0.4*	0.87 ± 0.02

Treatment	Female (*n*)	Male (*n*)
(b)		
Control	17.9 ± 1.1 (26)	1.3 ± 0.1 (37)
NP10	15.0 ± 1.6(24)	1.1 ± 0.1 (28)
NP100	13.9 ± 1.9 (8)	1.5 ± 0.1 (25)
EE1	16.2 ± 1.2 (26)	1.1 ± 0.1 (38)
EE10	16.7 ± 5.3 (5)	1.0 ± 0.1 (58)*
EE1 + NP10	18.9 ± 1.8 (11)	1.4 ± 0.1 (28)
EE1 + NP100	17.9 ± 1.6 (12)	1.3 ± 0.1 (27)
EE10 + NP10	N/A	1.4 ± 0.1 (47)
EE10 + NP100	15.1 ± 2.8 (5)	1.1 ± 0.1 (67)

Data are mean ± SEM. Significantly different from control group: * $P < 0.05$.

3.10 Adult Gonadal HSP70 Expression

There were no significant differences in the levels of gonadal HSP70 expression among treatments in adult zebrafish (Fig. 3.9).

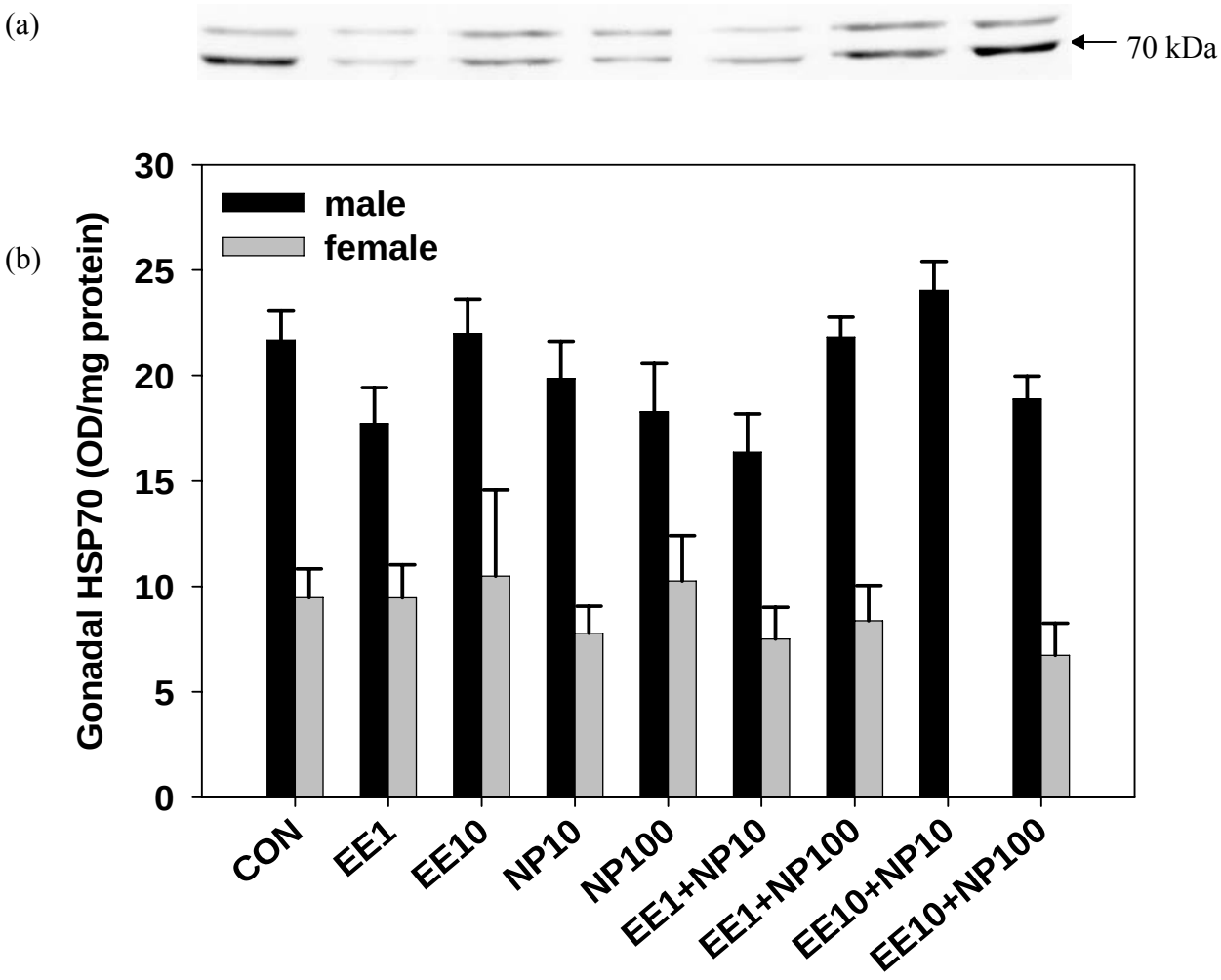


Figure 3.9 Gonadal heat shock protein 70 (HSP70) expression of adult zebrafish. Gonadal heat shock protein 70 expression in adult zebrafish previously exposed to 10 or 100 µg/l nonylphenol (NP10 or NP100 respectively), 1 or 10 ng/l 17α-ethinylestradiol (EE1 or EE10), 1 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE1+NP10), 1 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE1+NP100), 10 ng/l 17α-ethinylestradiol + 10 µg/l nonylphenol (EE10+NP10), or 10 ng/l 17α-ethinylestradiol + 100 µg/l nonylphenol (EE10+NP100), or solvent control (CON, 0.01% acetone v/v) from 2 to 60 days post-hatch. (a) Representative Western blot of HSP70 expression in adult zebrafish gonadal tissues. Lanes 1, 3, 5 and 7: CON; lane 2: EE10+NP100; lanes 4 and 6: EE1+NP100; (b) Densitometry of immunoreactive bands. Data are mean optical density (OD) ± S.E.M, one-way ANOVA followed by Tukey's test.

CHAPTER 4
DISCUSSION

4.1 Effects of Ethinylestradiol and Nonylphenol on 60 Days Post-hatch Zebrafish

It is generally assumed in aquatic ecotoxicology investigations that mixtures of xenoestrogens act in an additive manner. The major finding of the present study was that NP, a weak ER agonist, can behave both additively and non-additively when in combination with a potent ER agonist, EE, at environmentally relevant concentrations. This was particularly evident with VTG induction, a direct gene expression product following ER activation, in 60 dph zebrafish. Upon closer examination with one-way ANOVA on the effect of different levels of NP (NP0, NP10, and NP100) at each level of EE (EE0, EE1, and EE10), it was found that at 1 ng/l EE, the addition of 10 µg/l NP (EE1+NP10) increased the VTG induction significantly when compared to EE1 alone, exhibiting additivity of effects as neither EE1 nor NP10 alone resulted in significant VTG induction when compared with the control. However, in the EE1+NP100 exposure group, where the NP concentration increased from 10 µg/l to 100 µg/l while keeping the EE concentration constant at 1 ng/l, the level of VTG observed was significantly lower than that of EE1+NP10, and statistically insignificant when compared with EE1. This demonstrated that during exposure of zebrafish to 1 ng/l EE, lower levels of NP (NP10) appeared to act additively with EE in terms of VTG induction, while higher levels of NP (NP100) appeared to antagonize the action of EE, inducing lower levels of VTG. The antagonistic effect of NP was even more pronounced with mixture groups containing the higher EE concentration (10 ng/l). Fish exposed to 10 ng/l of EE + 100 µg/l of NP had a significantly lower VTG level when compared with the EE10 + NP10 group and the EE10 alone group.

One possible explanation for the observed non-additivity at certain combinations of EE + NP is that the EE and NP molecules are simply adhering to the mass action law in receptor binding. Using environmentally relevant concentrations where NP generally occurs at approximately ≥ 1,000 times greater concentrations than EE, the higher concentration of NP would theoretically allow NP a better chance of competing with EE for the available ER binding sites. Consequently, in binary mixtures of EE+NP at environmentally relevant levels, the addition of NP would allow NP to bind to some of the ER that would otherwise have been occupied by EE if EE was acting alone. The increase in NP concentration would therefore increase the proportion of ERs bound to NP. Since NP has been shown to be a weak or partial ER agonist, meaning that it binds to ER but produces a diminished response (e.g. VTG induction) compared to a full agonist like EE, the lower overall response induced by the binary mixture of NP and EE would be due to NP's weaker estrogenicity in comparison to EE. Therefore, NP may not be acting as an ER antagonist in the classical sense, i.e. binding to the ER and inhibiting ER-dependent responses. However, by its very nature as a weak or partial ER agonist, it may be lowering ER-dependent responses in binary mixtures of NP and EE where its concentration is high enough that it can displace EE binding to the ER, resulting in, in this case, lower VTG induction than when EE was acting alone. This hypothesis also allows us to explain the additive effect at a lower EE + NP concentration (EE1 + NP10), since at these lower EE + NP concentrations, ER are not as saturated, allowing all EE and NP molecules to bind and elicit each compound's respective level of estrogenicity. It is important to note however that the decreased VTG associated with increasing NP concentration in treatments containing 10 ng/l of EE (EE10, EE10+NP10 and EE10+NP100) is relative within those groups: the addition of NP to

10 ng/l of EE did not decrease VTG levels lower than that observed in groups containing 1 ng/l of EE (EE1, EE1+NP10 and EE1+NP100).

There are few studies that have examined *in vivo* xenoestrogen mixture toxicity in fish. Nonylphenol was reported to act additively in binary mixtures with E2, at concentrations below their individual LOECs, on VTG induction in juvenile female rainbow trout (*Oncorhynchus mykiss*) (Thorpe et al., 2001). Despite possible differences in species sensitivity to NP, lifestage sensitivity (3 months old vs. newly hatched), sex (female vs. mixed population) and duration of exposure (14 vs. 60 days), the result by Thorpe et al. (2001) is in agreement with our low EE + NP (EE1 + NP10) data where exposure to each compound alone did not induce significant increases in VTG, while combination of the two resulted in a significant increase. It is also possible that the discrepancy in the observed effect of binary xenoestrogen mixtures between the two studies may be due to the different source of NP utilized in each study. It has been shown that technical grade NP contains approximately 20 *p*-substituted isomers; each possessing their own estrogenic potency. Using the MVLN cell assay, a method which determines ER binding affinity via MCF-7 human breast carcinoma cells transfected with an ER controlled luciferase reporter gene, it was established that the estrogenicity of the studied isomers were not equal; the relative potency was found to be: p353-NP > p33-NP = p363-NP > p252-NP > P22-NP = P262-NP (Preuss et al., 2006). Therefore, with manufacturers producing NP mixtures containing their own relative proportions of isomers, the overall estrogenicity of technical grade NP from two companies could differ greatly from one another. For instance, the same study also utilized the MVLN cell assay to assess the relatively estrogenicity of two commercially available technical p-NP mixtures, p-NP Fluka (85% purity, Fluka Germany) and p-NP Acros (99% purity, Aldrich

Germany) It was demonstrated that the Aldrich mixture was about 1.18 times more potent than the Fluka mixture (Preuss et al., 2006).

Furthermore, in addition to being a weak ER agonist, NP has been shown to interact with cytochrome P450 enzymes such as the CYP1A subfamily in fish (Arukwe et al., 1997a; Hasselberg et al., 2005). The expression of CYP1A1 is regulated by aryl hydrocarbon receptor (AhR) through which some AhR agonists have been shown to be anti-estrogenic (Hahn, 2002). As well, recent reports have shown that some AhR agonists directly induce estrogenic activity through AhR-ERα crosstalk (Abdelrahim et al., 2006; Liu et al., 2006; Shipley and Waxman, 2006). By exposing juvenile Atlantic salmon (*Salmo salar*) to nominal concentrations of 5, 15, 50 µg/l NP or ethanol control statically for 7 days, Meucci and Arukwe (2006) reported that CYP1A1 and AhR mRNA levels in the 5 and 15 µg/l NP-exposed groups were temporally decreased at day 3 post-exposure while significantly induced at day 7, compared to the control group. In fish exposed to 50 µg/l NP, CYP1A1 mRNA levels were decreased at days 3 and 7. In spite of the decreased CYP1A1 mRNA levels at day 7, AhR mRNA was significantly increased (Meucci and Arukwe, 2006). The aforementioned studies highlight the complex nature of NP's interaction with different receptor (signal transduction) pathways and the resulting difficulty involved with predicting its estrogenicity.

Recent studies that have examined xenoestrogen mixture toxicity in other species or tissues and have also reported departure from additivity of responses. Xie et al. (2005) utilized a rainbow trout VTG assay to evaluate the estrogenicity of four herbicides, two alkylphenol ethoxylate-containing surfactants, and binary mixtures of herbicides with the surfactants. They observed that 2,4-D alone displayed estrogenic activity via VTG induction and in binary mixtures with target prospreader activator (TPA), an alkylphenol ethoxylate -containing

surfactant, exhibited greater than additive VTG response at the lowest concentrations tested, but a less than additive response at the highest combined concentrations. However, the results are complicated by the fact that 2,4-D contains trace quantities of several dioxin-like compounds which may bind to AhR and interfere with the overall estrogenic response. In addition, the surfactant TPA may contain components other than alkyphenol ethoxylate that can also affect the observed estrogenic response. Another recent study (Rajapakse et al., 2004) utilized the *in vitro* E-SCREEN assay to evaluate mixture toxicity of six xenoestrogens: E2, EE, NP, octylphenol (OP), genistein and bisphenol A. E-SCREEN measures the effect of these chemicals on the proliferation of estrogen-dependent MCF-7 human breast cancer cells. It was demonstrated that the presence of NP and OP was associated with the antagonism observed in five- and six-component mixtures. It should be noted, however, that the endpoint measured via E-SCREEN may not necessarily be exclusively estrogenic as the number of cancer cells produced within a given amount of time takes into account the number of cells produced through ER activation as well as cells lost through cytotoxicity. The authors reported that higher concentrations of NP and OP were accompanied by a marked decrease in cell numbers. The decrease may be attributed to cytotoxicity, and may or may not be ER-mediated as there are numerous ways in which chemicals can exert growth-restricting or cytotoxic effects (Rajapakse et al., 2004).

An additional factor to consider when evaluating the nature of the interaction(s) between compounds in mixture toxicity studies is that the type of interaction may be dependent on the particular biological response(s) measured. Teles et al. (2004) investigated the effect of binary mixtures of β–naphthoflavone (BNF), an AhR agonist, combined with either E2 or NP on juvenile sea bass (*Dicentrarchus labrax*). Their endpoints included biotransformation responses by measuring liver cytochrome P450 content (P450), ethoxyresorufin-O-deethylase (EROD), and

glutathione S-transferase (GST) activities, effects on the endocrine function via determination of plasma cortisol and glucose levels, and genotoxicity through assessing the erythrocytic nuclear abnormalities (ENA) frequency. It was demonstrated that with respect to plasma glucose concentration, a synergistic interaction was evident between BNF and NP, while sporadic antagonism was found between BNF and E2 after a 4h exposure. Liver EROD activity was not significantly altered by single E2 or NP exposure, however, when combined with BNF, both were able to induce EROD activity. While NP exposure alone was able to significantly increase liver P450 content, its action appeared to be antagonized with the addition of BNF. As well, upon evaluating genotoxicity, single E2 exposure did not induce an ENA increase, while mixtures of E2+BNF displayed a significant induction (Teles et al., 2004). Based on these observations, it is important to be aware that the type of interaction between compounds in mixture toxicity studies may differ depending on the particular biological response(s) measured. In particular, responses closely linked with gene expression, such as VTG induction, will likely produce different patterns of mixture toxicity when compared to more complex, integrative responses such as reproduction. A similar trend of non-additivity in the present study was observed in certain responses determined in the adult breeding experiments. Specifically, exposure to NP100 in the presence of EE1 abolished the decreased mean egg production observed with EE1 alone. Similarly, in the EE10 exposure groups, the addition of 100 µg/l NP (EE10 + NP100) resulted in egg production that was not significantly different from EE10 alone. These results collectively suggest that lower levels of NP (NP10) may have acted additively in the presence of 10 ng/l EE, while higher levels of NP (NP100) did not further reduce the mean egg production, instead, resulted in mean egg production that was similar to EE10.

4.2 Reproductive Studies of 240 Days Post-hatch Zebrafish

Results from the breeding trials also highlighted the toxicity of early life stage exposure to 10 ng/l of EE in zebrafish. After a 6 month period of depuration, there was a persistent impact on several reproductive parameters. Treatments containing 10 ng/l of EE (EE10, EE10+NP10 and EE10+NP100) resulted in the lowest cumulative number of eggs produced during the duration of the breeding trial, and the same three groups possessed the three lowest percentages of successful breeding trials. Other studies investigating the breeding success in zebrafish exposed during sexual differentiation to environmentally relevant concentrations of xenoestrogens have reported a similar reduction in reproductive fitness. Hill and Janz (2003) exposed zebrafish from 2 to 60 dph to nominal concentrations of 10–100 µg/l of NP or 1–100 ng/l of EE, then raised in clean water from 60 to 120 dph, and reported that the NP–100 µg/l group had decreased egg hatchability and swim–up success, while EE–10 ng/l resulted in decreased egg viability and hatchability as well as swim–up success. Another study reported that the exposure of zebrafish embryos to nominal concentrations of 10 or 25 ng/l of EE until 90 days post–fertilization (dpf) and allowed to recover in clean water for 5 months, resulted in a reduced number of spawning females as well as reduced egg production (Van den Belt et al., 2003). Fenske et al. (2005) evaluated the reproductive capacities of zebrafish exposed to 3 ng/l EE from either 0–42 dpf follow by 76 d depuration or 0–118 dpf followed by 58 d depuration. They reported that there were no significant effects on the reproductive fitness of the fish exposed from 0–42 dpf, which differs from present study where exposure to a lower EE concentration (1 ng/l) from 2–60 dph, followed by six months of depuration, resulted in a significant decrease in egg viability. However, this may be explained by the difference in the duration of exposure, and is supported by their finding that zebrafish exposed for a longer period, from 0–118 dpf, had decreased egg production and fertilization success (Fenske et al., 2005). This further indicates

the need to account for the length of exposure in assessing the effects of xenoestrogens, particularly when overlapping the period of sexual differentiation and gametogenesis.

Other fish models such as medaka (*Oryzias latipes*) and fathead minnow (*Pimephales promelas*) have also been utilized in similar investigations. Medaka exposed to 0.2 or 2 ng/l of EE from 2-5 dph until sexual maturity (between four and six months of age) displayed normal mating behavior and reproductive success, while males exposed to 10 ng/l of EE exhibited suppressed reproductive behavior, and females had poor reproductive success (Balch et al., 2004). In fathead minnow exposed to 0.2 or 1.0 ng/l of EE from 0–301 dph, no effects on female egg production were reported (Lange et al., 2001). The variation seen in estrogenic sensitivity highlights the importance of taking into account species differences when evaluating the effects of xenoestrogen exposure in fish.

The incorporation of fecundity evaluation in studies examining the impact of xenoestrogen exposures during early development is regarded as a crucial step forward towards a better understanding of the extent of effects. However, caution should be noted when measuring reproductive endpoints such as daily egg production since natural variation in fecundity exists, as seen in the present as well as other studies (Lange et al., 2001; Brion et al., 2004; Nash et al., 2004). This emphasizes the need for multiple replications to minimize this inherent variability. Further caution should be observed when attempting to correlate the reduction in reproductive success to early life stage xenoestrogen exposures. Unlike VTG induction, the measurement of reproductive fitness is not a direct estrogen-specific response, but rather an integrative one involving not only the endocrine and reproductive systems. Other factors such as the physiological and health status of the fish could have critical implications. This is especially true in studies where fish are allowed a depuration period after inappropriate exposure to

xenoestrogens during early development. In these studies, the fish appeared to recover and continued with normal gonadal development with no apparent differences in GSI, yet decreased in reproductive success was still detected (Hill and Janz, 2003; Van den Belt et al., 2003; Brion et al., 2004). The present study observed similar results, and with the exception of the significantly lowered GSI in EE10 adult males, no differences in GSI from any treatments were observed when compared with the control. Several authors have suggested that a disruption in mating behaviour could help explain such reproductive failure (Gray et al., 1999; Bjerselius et al., 2001; Balch et al., 2004), however, behavioural changes may or may not be a direct effect of xenoestrogen exposures.

4.3 Sex Distribution and Gonad Development in Zebrafish

The results from the reproduction experiments in the present study were partly explained upon subsequent determination of the adult sex distribution. The EE10+NP10 group had only one female at the end of the reproductive trials, which most likely contributed to the observed lack of egg production from that group. Two other groups that experienced significantly decreased mean egg production per breeding trial when compared with the control group also contained lower numbers of females, 7.8% (n=5) in EE10 and 6.9% (n=5) in EE10+NP100, when examined after completion of the reproductive studies. Interestingly, these three groups with the lowest female:male ratios and lowest mean egg production per breeding trial were the same groups that did not present any visible gonadal tissues during the 60 dph histological examination. The EE1-exposed fish also produced a significantly lower mean number of eggs per breeding trial, but the sex distribution was not significantly different from that of the control group. A similar trend of lower mean egg production per breeding trial and skewed sex distribution towards males, although neither was statistically significant, was also observed in the NP100 group.

The sex distribution of all treatment groups, excluding the EE10-containing groups which had no discernable gonadal tissues, were all skewed towards more females at the 60 dph histological examination. However, histological examination after the adult breeding trials revealed that the sex distribution of these groups did not deviate significantly from the control group. From these observations, it appears that when zebrafish are exposed during early development to concentrations of xenoestrogens that are below 10 ng/l EE, namely the groups EE1, NP10, EE1+NP10, and EE1+NP100, the effects of xenoestrogen exposure on sexual differentiation are reversible following a period of depuration. Other studies have reported a similar trend, where sex distribution of zebrafish exposed to xenoestrogens during early development was skewed towards more female, but more males developed after cessation of xenoestrogen exposures (Hill and Janz, 2003; Fenske et al., 2005). One possible explanation for this observation may lie in the nature of zebrafish gonad differentiation. Zebrafish are undifferentiated gonochorists whereby all gonads begin development as immature ovaries consisting solely of oogonia and primary growth stage oocytes (Takahashi, 1977). Eventually, some will continue their development into mature ovaries while the rest differentiate into testes (Takahashi, 1977; Uchida et al., 2002). Although the exact nature of what triggers the differentiation of the early immature ovaries into either mature ovaries or testes is not yet clear, increasing evidence is suggesting that it may be influenced by endogenous androgen and estrogen levels. Studies in fish have shown that inhibition of the cytochrome P450 aromatase complex, the enzyme responsible for converting androgens into estrogens, during early differentiating stages can lead to development as phenotypic males in genetic females (Piferrer et al., 1993; Kitano et al., 2000; Kroon and Liley, 2000). In zebrafish, the presence of sex steroids has been shown to induce gonadal feminization or masculinization after manipulation of

aromatase (Fenske and Segner, 2004). Therefore, it is likely that the gonadal development of genetic males during the early stage were suppressed as a result of xenoestrogen exposures, and after a period of depuration, the gonads of these males were able to resume their differentiation into testes. This explains the observation of apparent all-female populations at 60 dph in EE1, NP10, EE1+NP10, and EE1+NP100, as they included both genetic females with developing ovaries as well as genetic males with arrested gonadal maturation.

To a more severe extent, with a higher concentration of EE, fish in groups containing 10 ng/l EE (EE10, EE10+NP10 and EE10+NP100) experienced an increased suppression of gonadal development in that no discernable gonadal tissues were present when examined at 60 dph. The lack of visible gonadal tissues could potentially be due to divesting the energy normally reserved for gametogenesis into VTG production, as these three groups had the highest VTG induction amongst all treatments. The sex distribution in the EE10 exposure groups at the end of the breeding experiments were all predominately male, which can be attributed to the possibility that females may be more sensitive to higher concentrations of xenoestrogens and especially chronic exposure, thus experiencing higher mortality. Generally, compared to male fish, females are more metabolically stressed due to the higher energy investment needed for egg production. With the additional stress of xenoestrogen exposures (i.e. as seen in the high EE exposure groups), it is possible that the females in groups containing 10 ngl/l of EE (EE10, EE10+NP10, and EE10+NP100) experienced further energy expenditure and depletion leading to their death. Unfortunately, this study was unable to sufficiently test the hypothesis of sex-specific sensitivity to xenoestrogen exposures. Due to the lack of prominent sexual dimorphism in zebrafish, gender is not easily determined by visual inspection, therefore during the depuration period, an accurate account of sex distribution could not be determined unless the fish were

examined histologically. As well, when a fish dies, its body cavity, especially the ovaries are quickly consumed by other fish soon after, often leaving very little evidence of their demise. Thus, if any carcasses were still left behind, the gender of the dead fish would have been very difficult to establish.

The plasticity of zebrafish gonadal development was demonstrated through the reversal of sex distribution seen with the adult zebrafish after being exposed from 2 to 60 dph to various concentrations of EE, NP and EE+NP and allowed a six month depuration. In addition, upon measuring the adult GSI, with the exception of the significantly lowered GSI in EE10 adult males, no differences in GSI from any treatments were observed when compared with the control. Despite this apparent recovery, further evidence of reduced reproductive fitness, other than the aforementioned decreased daily and overall egg production, were observed. Eggs produced by fish in groups EE10, EE1+NP100 and EE10+NP100 experienced decreased viability when compared with the control. Decreased hatchability in EE10 was also observed. As well, the swim-up success of the F1 generation from groups EE10, EE1+NP10, EE1+NP100 and EE10+NP100 was significantly reduced. This illustrates that inappropriate exposure to xenoestrogens during the critical period of gonadal development and sexual differentiation, even after a long period of depuration, may have irreversible effects on the reproductive system. Further research is warranted to elucidate the mechanism(s) of both the cause of the reproductive failure and the transgenerational effects.

4.4 HSP70 and Apoptosis

Another biochemical endpoint examined in the current study was the expression of HSP70 in whole body juveniles and adult gonads to investigate the feasibility of correlating HSP70 responses with estrogenic exposures in zebrafish. There were no significant differences between treatments in the level of whole body HSP70 expression in the 60 dph fish. Although

some studies have reported elevated levels of piscine HSP70 as well as other heat shock proteins associated with exposure to various environmental stressors including heavy metals, industrial effluents, pesticides, and polycyclic aromatic hydrocarbons, it is noteworthy that the majority of studies evaluated the response in cell lines, primary cell cultures or specific tissues and not of the whole animal (Basu et al., 2002). Other studies have indicated that the HSP response can differ among tissues (Smith et al., 1999; Rabergh et al., 2000), which may help explain the lack of significant differences in whole body HSP70 levels between the groups in this study since the measurement accounted for not only specific tissue types but the whole animal. There were also no significant differences between treatments in the gonadal HSP70 expression of the adult zebrafish at the end of the reproductive study. It is likely that 6 months of depuration after the 60 day exposure period was sufficient time for HSP70 to return to basal levels. Based on these results, whole body HSP70 does not appear to be a viable biomarker for chronic xenoestrogen exposure due to possible variations among tissues.

Originally, juvenile HSP70-eGFP zebrafish were proposed to be exposed to EE, NP, EE+NP or control from 2 to 60 dph in a parallel experiment with juvenile wild-type zebrafish. Unfortunately, due to the poor reproductive performance of the adult HSP70-eGFP transgenic zebrafish broodstock, no juvenile transgenic zebrafish were available for the exposure study. Otherwise, it would have served as a valuable model to determine if exposure to xenoestrogen(s) induces any tissue-specific HSP70 expression. This study also proposed to evaluate gonadal cell death via TUNEL staining, however, since excessive amount of sections were taken for H&E staining from the preserved samples, the majority of samples did not have sufficient gonadal tissues remaining for TUNEL. This prevented the effort to determine if the observed adverse effects from xenoestrogen exposure were linked to stress on the reproductive system.

Nevertheless, the whole body HSP70 results should serve as a basis for concentrating future efforts on examining tissue-specific HSP70 expression as well as gonadal apoptosis in order to better understand the mechanism(s) of the observed reproductive toxicity associated with exposure to xenoestrogens.

4.5 Duration of Exposure

The length of exposure for the present study was set at 60 days. Previous studies have demonstrated that 60 dph is sufficient time for the complete sexual differentiation in zebrafish. Takahashi (1977) reported that complete sex reversal and testicular formation occurs by 40 dph. Uchida et al. (2002) also reported a gonad transformation period 3-4 weeks after hatching, with the total disappearance of all oocytes in genetic males occurring by the end of 4 weeks. However, in the current study, 18% of the control fish at 60 dph possessed undifferentiated gonads. Andersen et al. (2004) reported similar results when studying the effects of the anti-estrogen ZM 189,156 and the aromatase inhibitor fadrozole on juvenile zebrafish. They described that at 60 dph, the water and solvent control groups had 14% and 24% of fish possessing undifferentiated gonads, respectively. Other studies have also reported the presence of undifferentiated gonads in control zebrafish at 60 dph (Hill and Janz, 2003; Orn et al., 2003). The variation observed in timing of the gonad maturation period between the studies may be attributed to strain differences and/or the different rearing conditions of each respective study (Orn et al., 2003). Maack et al. (2003) suggested that stocking density, feeding conditions, social factors and water temperature, and not strain differences, may affect the timing of sexual development in zebrafish. It is also possible that the particular batch of fish used in the present study might not have been fully developed at 60 dph, as Maack et al. (2003) observed that high inter-individual variability exists in the timing of sex differentiation within each strain with no obvious correlation to body mass. This appears to be the most likely explanation for the present

study as the body mass of the control fish at 60 dph was similar to the control fish in Van Den Belt et al. (2003), while less than the controls in Andersen et al. (2004), Hill and Janz (2003), and Orn et al. (2003), with no apparent correlation to each respective level of undifferentiated gonads. In the present study, we also observed the lack of visible gonad tissues at 60 dph in fish from exposure groups containing 10 ng/l of EE (EE10, EE10+NP10 and EE10+NP100). Andersen et al. (2003) also observed 60 dph zebrafish with no visible gonads when exposed to 15.4 ± 1.4 ng/l EE from hatch to 60 dph. As well, Van Den Belt et al. (2003) reported a concentration-dependent increase in the number of fish with no discernable gonads when exposed to 0.1, 1, 10 or 25 ng/l of EE from hatch until 3 months post-hatch. One possible explanation, as mentioned above, could be that the energy required for VTG production (and potentially other ER-dependent gene products) is quite high, thus the energy normally devoted to gonad development may be diverted to VTG synthesis. Consequently, groups exhibiting high VTG induction would yield more individuals with small or underdeveloped gonads. These results suggest that the delay in the maturation process associated with EE exposures in zebrafish is both concentration- and duration- dependent.

CHAPTER 5
CONCLUSIONS

In summary, the current results, derived from exposing zebrafish to binary mixtures of the xenoestrogens EE and NP from 2 to 60 dph, demonstrated that:

- Depending on relative exposure concentrations, the effect of binary mixtures of EE + NP on VTG induction can be additive or non-additive. Higher levels of NP in the presence of EE appeared to antagonize the action of EE, inducing lower levels of VTG than when EE was acting alone.
- Similar non-additive effects were observed with mean number of eggs produced per breeding trial from adult fish that were allowed to recover in clean water for 6 months after the exposure experiment. Fish in the EE1 exposure group produced significantly lower number of eggs compared with the control group while the addition of NP in EE1+NP10 and EE1+NP100 exposure groups resulted in egg production that were not significantly different from the control group.
- Examination of the adult fish after the breeding trials revealed that groups previously exposed to 10 ng/l of EE (EE10, EE10+NP10 or EE10+NP100) had sex distribution that skewed towards more males. We hypothesize this may be the result of female zebrafish being more sensitive towards chronic xenoestrogen exposures, and in the process accruing more stress, leading to subsequent death.
- Despite the recovery of zebrafish in terms of sex distribution, GSI, and condition factor, in NP100, EE1, EE1+NP10 and EE1+NP100, breeding trials revealed

significant reductions in egg viability and hatchability as well as F1 swim-up success. These results suggest persistence of impairment in reproductive fitness.

- Sixty days may not be of sufficient time to encompass the completion of zebrafish gonad maturation; especially in xenoestrogen studies where those compounds have been demonstrated to interfere with / delay the maturation process.
- Overall, based on the results of this study it is recommended that caution be exercised in ecological risk assessments when assuming additivity of fish responses to xenoestrogen mixtures.

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APPENDIX A
JUVENILE ZEBRAFISH CARE

1 *Coleps* Infestation

Originally, fertilized eggs were incubated in plastic Petri dishes (50 eggs per dish) filled with dechlorinated tap water. Subsequent to hatching, fry were fed finely-crushed Nutrafin Max flake food. It was noted that the number of fry would decrease dramatically over a period of 7 to 10 days. In most cases, no signs of their demise were present; it appeared that the fry had simply vanished. By 14 dph, the mortality rate was 100%. Upon closer inspection using a dissecting microscope, the presence of coleps was recorded. *Coleps* are free-living freshwater protozoa that are known to swarm freshly hatched fry and consume its body in as little as 1 h, leaving virtually no evidence of the attack. The infestation of *Coleps* was eliminated by incubating the eggs and raising the fry in sterilized egg water.

2 Barrier vs. No-Barrier

After the implementation of egg water usage for incubation, the mortality rate of fry was not as dramatic as when the fry were incubated in dechlorinated tap water (62.1 ± 4.1% vs. 11.4 ± 1.7% at 7 dph), nonetheless, the survival rate of fry remained suboptimal (12.1 ± 2.0% at 21dph). It was hypothesized that perhaps the reduced survival rate of fry were due to stress induced by being housed in Petri dishes which are transparent, thus were subjected to disturbances caused by experimenters performing daily maintenance of the environmental chamber. Cylindrical-shaped visual barriers were constructed out of paper and placed around some Petri dishes to examine if; 1) the observed suboptimal survival rate was brought on by stress due to visual stimulus, and 2) if the survival rate would increase upon placement of the

barrier. There was no improvement in survival rate from groups with the barrier when compared to groups without the barrier at 21 dph (17.6 ± 1.9% vs. 23.2 ± 3.1% respectively).

3 Feeding Regimen

Further investigation revealed that fry were not eating the finely-crushed Nutrafin Max flake food, resulting in starvation and eventual mortality. Since the newly hatched fry are quite transparent in appearance, the lack of food intake into a fry's stomach can be easily observed by the naked eye. This hypothesis of lack of food intake offered a possible explanation for the gradual mortality rate, unlike the rapid mortality rate experienced with Coleps infestation. Several feeding regimen were employed to determine the optimum diet that ensures high survival rate.

3.1 Microencapsulated Feed

A microencapsulated powder feed specifically formulated for larval fish was gifted by Dr. Patrick Krone of the Department of Anatomy and Cell Biology at University of Saskatchewan. Prior to feeding, the powder feed was mixed with egg water to create a slurry solution. Using a Pasteur pipette, a drop of the solution was fed to each Petri dish. It did not appear that the fry were eating the food as the survival rate remained suboptimal at 21 dph (23.9 ± 2.9%). Furthermore, the slurry caused the incubation water to become cloudy, necessitating a water change after each feeding, which proved unfeasible since the 60 dph exposure experiment proposed to have a water change every 48 h to minimize stress on the larval fish due to handling.

3.2 Hard-Boiled Chicken Egg Yolk

Hard-boiled egg yolk was mixed with egg water to make a slurry solution similar to the microencapsulated powder feed/egg water solution. The results were similar to the microencapsulated feed (25.3 ± 2.8% survival at 21 dph), with fish not eating the food as well as

requiring a water change after each feeding. The survivorship of the larval zebrafish remained suboptimal with this diet.

3.3 *Paramecium multimicronucleatum* and Brine Shrimp (*Artemia franciscana sp.*)

As outlined in Chapter 2, the optimum feeding regimen was the combination of *Paramecium multimicronucleatum* and freshly hatched brine shrimp. At 2 dph, using a Pasteur pipette, 2 drops of paramecium culture were fed to each Petri dish twice daily. As mentioned previously, due to the transparent appearance of larval zebrafish, it was possible to monitor their food intake by observing them with the naked eye. The paramecium cultures maintained for this study were green in color, thus paramecium intake by the fry was characterized by green rotund abdomen. At 7 dph, the larval zebrafish were fed a combination of paramecium (twice daily) and freshly hatched brine shrimp (once daily). One hour after the brine shrimp feeding, uneaten brine shrimp were removed from the Petri dish. The mixed diet was maintained from 7 to 14 dph. By 14 dph most, if not all, were able to eat brine shrimp. By 21dph, the mean survival rate of the 15 batches tested was 83.2 ± 2.3 %.

To summarize, in conjunction with incubating in sterile egg water, a mixture of two live foods, paramecium and brine shrimp, were demonstrated to yield better survival rate of larval zebrafish than the other diets tested.

APPENDIX B
JUVENILE ZEBRAFISH MAINTENANCE AND CARE STUDY DATA

Treatment: incubation with dechlorinated tap water, fed finely crushed Nutrafin Max flake food

Trials	Total # of fry at 0 dph	Total # of fry at 7 dph	% Survival at 7 dph
1	542	32	5.9
2	165	2	1.2
3	426	46	10.8
4	275	34	12.4
5	674	89	13.2
6	648	124	19.1
7	134	24	17.9
8	516	37	7.2
9	846	126	14.9
10	325	59	18.2
11	812	184	22.7
12	273	35	12.8
13	385	0	0.0
14	276	25	9.1
15	297	16	5.4

By 14 dph, mortality rate for all trials were 100%.

Treatment: incubation with egg water, fed finely crushed Nutrafin Max flake food

Trials	Total # of fry at 0 dph	Total # of fry at 7 dph	% Survival at 7 dph	Total # of fry at 21 dph	% Survival at 21 dph
1	250	170	68.0	15	6.0
2	310	213	68.7	25	8.1
3	231	115	49.8	14	6.1
4	340	232	68.2	56	16.5
5	315	178	56.5	16	5.1
6	369	271	73.4	61	16.5
7	643	457	71.1	0	0.0
8	410	168	41.0	126	30.7
9	583	327	56.1	82	14.1
10	618	294	47.6	41	6.6

11	519	149	28.7	72	13.9
12	632	521	82.4	125	19.8
13	310	187	60.3	53	17.1
14	549	396	72.1	73	13.3
15	815	715	87.7	61	7.5

Treatment: incubation with egg water, fed finely crushed Nutrafin Max flake food

Trials	Total # of fry at 0 dph	Total # of fry at 7 dph	% Survival at 7 dph	Total # of fry at 21 dph	% Survival at 21 dph
1	250	170	68.0	15	6.0
2	310	213	68.7	25	8.1
3	231	115	49.8	14	6.1
4	340	232	68.2	56	16.5
5	315	178	56.5	16	5.1
6	369	271	73.4	61	16.5
7	643	457	71.1	0	0.0
8	410	168	41.0	126	30.7
9	583	327	56.1	82	14.1
10	618	294	47.6	41	6.6
11	519	149	28.7	72	13.9
12	632	521	82.4	125	19.8
13	310	187	60.3	53	17.1
14	549	396	72.1	73	13.3
15	815	715	87.7	61	7.5

Treatment: incubation with egg water, fed finely crushed Nutrafin Max flake food &paper barrier

Trials	Total # of fry at 0 dph	Total # of fry at 21 dph	% Survival at 21 dph
1	78	7	9.0
2	159	34	21.4
3	194	50	25.8
4	195	32	16.4
5	163	39	23.9
6	180	20	11.1
7	126	19	15.1
8	74	8	10.8
9	61	11	18.0
10	129	31	24.0

Treatment: incubation with egg water, fed finely crushed Nutrafin Max flake food & no paper barrier

Trials	Total # of fry at 0 dph	Total # of fry at 21 dph	% Survival at 21 dph
1	185	51	27.6
2	116	11	9.5
3	80	15	18.8
4	211	57	27.0
5	50	10	20.0
6	74	14	18.9
7	80	7	8.8
8	172	54	31.4
9	103	42	40.8
10	214	63	29.4

Treatment: incubation with egg water, fed microencapsulated powder feed

Trials	Total # of fry at 0 dph	Total # of fry at 21 dph	% Survival at 21 dph
1	189	11	5.8
2	421	142	33.7
3	396	48	12.1
4	368	124	33.7
5	319	86	27.0
6	374	36	9.6
7	371	90	24.3
8	265	104	39.2
9	282	19	6.7
10	209	39	18.7
11	311	66	21.2
12	172	58	33.7
13	272	71	26.1
14	419	137	32.7
15	380	127	33.4

Treatment: incubation with egg water, fed crushed hard-boiled egg yolk

Trials	Total # of fry at 0 dph	Total # of fry at 21 dph	% Survival at 21 dph
1	213	12	5.6
2	171	46	26.9
3	322	125	38.8
4	206	31	15.0
5	144	17	11.8
6	115	37	32.2

7	162	42	25.9
8	334	107	32.0
9	237	76	32.1
10	135	16	11.9
11	330	127	38.5
12	210	64	30.5
13	134	35	26.1
14	185	27	14.6
15	182	67	36.8

Treatment: incubation with egg water, fed paramecium and freshly hatched brine shrimp

Trials	Total # of fry at 0 dph	Total # of fry at 21 dph	% Survival at 21 dph
1	186	156	83.9
2	235	187	79.6
3	201	165	82.1
4	248	231	93.1
5	103	67	65.0
6	328	284	86.6
7	110	95	86.4
8	238	197	82.8
9	203	184	90.6
10	263	167	63.5
11	113	100	88.5
12	94	85	90.4
13	156	124	79.5
14	317	297	93.7
15	219	180	82.2

APPENDIX C
SIXTY DAYS POST-HATCH DATA

Treatment: Control

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
5-1-1	15.12	21	0.61
5-1-2	15.94	25	0.62
5-1-3	12.18	12	0.66
5-1-4	15.18	19	0.54
5-1-5	16.88	28	0.58
5-1-6	14.14	16	0.57
5-1-7	12.67	14	0.69
5-1-8	11.00	10	0.75
5-1-9	14.89	21	0.64
5-1-10	11.97	10	0.58
5-1-11	11.97	12	0.70
5-1-12	10.63	7	0.58
5-1-13	15.99	24	0.59
5-1-14	11.64	12	0.76
5-2-1	14.29	19	0.65
5-2-2	9.00	5	0.69
5-2-3	11.18	9	0.64
5-2-4	11.53	11	0.72
5-2-5	9.63	6	0.67
5-2-6	8.90	6	0.85
5-2-7	12.71	12	0.58
5-2-8	9.52	8	0.93
5-2-9	12.00	12	0.69
5-2-10	12.79	14	0.67
5-2-11	13.77	15	0.57
5-2-12	10.27	8	0.74
5-2-13	14.96	21	0.63
5-2-14	15.36	25	0.69
5-3-1	9.54	7	0.81
5-3-2	12.82	14	0.66
5-3-3	10.85	10	0.78
5-3-4	10.19	6	0.57
5-3-5	13.19	14	0.61
5-3-6	13.89	15	0.56
5-3-7	13.89	19	0.71
5-3-8	10.76	9	0.72
5-3-9	10.17	7	0.67
5-3-10	12.41	13	0.68
5-3-11	13.75	17	0.65

5-3-12	12.81	12	0.57
5-3-13	11.17	9	0.65
5-3-14	12.42	11	0.57

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: NP10

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
3-1-1	18.24	36	0.59
3-1-2	14.84	19	0.58
3-1-3	11.89	10	0.59
3-1-4	14.58	19	0.61
3-1-5	14.58	18	0.58
3-1-6	14.58	19	0.61
3-1-7	15.57	22	0.58
3-1-8	10.35	7	0.63
3-1-9	12.55	12	0.61
3-1-10	11.38	10	0.68
3-1-11	13.51	14	0.57
3-1-12	14.16	16	0.56
3-1-13	12.50	12	0.61
3-1-14	13.15	16	0.70
3-2-1	12.54	14	0.71
3-2-2	13.82	19	0.72
3-2-3	13.82	17	0.64
3-2-4	14.21	19	0.66
3-2-5	15.51	22	0.59
3-2-6	15.51	22	0.59
3-2-7	13.45	17	0.70
3-2-8	11.88	15	0.89
3-2-9	10.72	9	0.73
3-2-10	13.75	18	0.69
3-2-11	12.52	11	0.56
3-2-12	11.33	8	0.55
3-2-13	12.67	14	0.69
3-2-14	9.67	7	0.77
3-3-1	10.65	9	0.75
3-3-2	12.21	12	0.66
3-3-3	13.65	18	0.71
3-3-4	14.72	18	0.56
3-3-5	15.25	21	0.59
3-3-6	14.45	17	0.56
3-3-7	10.39	9	0.80
3-3-8	12.77	13	0.62
3-3-9	13.91	15	0.56
3-3-10	14.41	20	0.67

3-3-11	16.75	26	0.55
3-3-12	13.39	13	0.54
3-3-13	10.93	9	0.69
3-3-14	12.62	15	0.75

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: NP100

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
4-1-1	17.09	29	0.58
4-1-2	8.44	40	6.65
4-1-3	15.67	23	0.60
4-1-4	14.71	19	0.60
4-1-5	13.08	13	0.58
4-1-6	14.22	19	0.66
4-1-7	11.75	11	0.68
4-1-8	12.97	12	0.55
4-1-9	14.19	20	0.70
4-1-10	10.23	5	0.47
4-1-11	12.84	13	0.61
4-1-12	12.70	14	0.68
4-1-13	20.10	53	0.65
4-1-14	18.45	34	0.54
4-2-1	12.39	12	0.63
4-2-2	10.27	6	0.55
4-2-3	14.17	18	0.63
4-2-4	13.90	16	0.60
4-2-5	11.98	13	0.76
4-2-6	14.14	20	0.71
4-2-7	9.69	6	0.66
4-2-8	8.38	4	0.68
4-2-9	9.54	6	0.69
4-2-10	12.91	17	0.79
4-2-11	14.73	21	0.66
4-2-12	11.91	10	0.59
4-2-13	11.91	11	0.65
4-2-14	13.10	15	0.67
4-3-1	14.38	19	0.64
4-3-2	13.62	16	0.63
4-3-3	12.04	11	0.63
4-3-4	14.66	21	0.67
4-3-5	9.87	6	0.62
4-3-6	14.40	18	0.60
4-3-7	10.43	8	0.71
4-3-8	12.96	14	0.64
4-3-9	9.36	5	0.61

4-3-10	13.75	17	0.65
4-3-11	14.94	23	0.69
4-3-12	10.27	8	0.74
4-3-13	17.29	33	0.64
4-3-14	13.32	15	0.63

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: EE1

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
1-1-1	10.12	7	0.68
1-1-2	14.97	21	0.63
1-1-3	12.03	10	0.57
1-1-4	15.51	22	0.59
1-1-5	11.44	8	0.53
1-1-6	13.29	14	0.60
1-1-7	15.55	22	0.59
1-1-8	14.15	17	0.60
1-1-9	13.01	14	0.64
1-1-10	12.77	12	0.58
1-1-11	11.02	8	0.60
1-1-12	12.09	10	0.57
1-1-13	13.79	16	0.61
1-1-14	12.92	14	0.65
1-2-1	10.50	8	0.69
1-2-2	14.50	20	0.66
1-2-3	14.00	18	0.66
1-2-4	11.00	10	0.75
1-2-5	12.00	12	0.69
1-2-6	12.00	13	0.75
1-2-7	12.00	10	0.58
1-2-8	12.00	10	0.58
1-2-9	11.50	11	0.72
1-2-10	11.00	7	0.53
1-2-11	14.00	17	0.62
1-2-12	14.00	16	0.58
1-2-13	10.50	8	0.69
1-2-14	13.00	15	0.68
1-3-1	14.00	17	0.62
1-3-2	15.00	20	0.59
1-3-3	12.00	13	0.75
1-3-4	14.00	16	0.58
1-3-5	12.00	12	0.69
1-3-6	14.00	17	0.62
1-3-7	13.00	14	0.64
1-3-8	13.00	13	0.59

1-3-9	13.00	13	0.59
1-3-10	11.00	9	0.68
1-3-11	14.00	16	0.58
1-3-12	11.50	10	0.66
1-3-13	11.00	8	0.60
1-3-14	9.00	15	2.06

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: EE10

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
2-1-1	9.43	6	0.72
2-1-2	10.46	8	0.70
2-1-3	9.77	6	0.64
2-1-4	10.51	9	0.78
2-1-5	11.03	9	0.67
2-1-6	9.62	7	0.79
2-1-7	9.62	7	0.79
2-1-8	8.84	5	0.72
2-1-9	9.75	7	0.76
2-1-10	9.37	6	0.73
2-1-11	8.67	5	0.77
2-1-12	8.00	3	0.59
2-1-13	7.70	3	0.66
2-1-14	8.69	5	0.76
2-2-1	11.00	10	0.75
2-2-2	10.50	9	0.78
2-2-3	7.92	3	0.60
2-2-4	8.85	5	0.72
2-2-5	9.71	7	0.76
2-2-6	9.83	12	1.26
2-2-7	10.23	8	0.75
2-2-8	10.20	8	0.75
2-2-9	7.53	5	1.17
2-2-10	8.42	5	0.84
2-2-11	8.90	7	0.99
2-2-12	8.22	6	1.08
2-2-13	8.00	5	0.98
2-2-14	8.00	7	1.37
2-3-1	10.50	10	0.86
2-3-2	11.50	11	0.72
2-3-3	8.00	4	0.78
2-3-4	8.00	4	0.78
2-3-5	9.00	6	0.82
2-3-6	8.00	5	0.98
2-3-7	9.00	6	0.82

2-3-8	7.00	3	0.87
2-3-9	9.00	5	0.69
2-3-10	8.00	4	0.78
2-3-11	9.00	6	0.82
2-3-12	8.00	5	0.98
2-3-13	7.00	5	1.46
2-3-14	10.00	9	0.90

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: EE1+NP10

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
6-1-1	16.16	28	0.66
6-1-2	16.63	29	0.63
6-1-3	15.26	21	0.59
6-1-4	11.34	9	0.62
6-1-5	11.66	10	0.63
6-1-6	10.83	8	0.63
6-1-7	14.06	19	0.68
6-1-8	13.55	14	0.56
6-1-9	12.35	13	0.69
6-1-10	13.25	15	0.64
6-1-11	11.36	12	0.82
6-1-12	13.66	19	0.75
6-1-13	10.00	7	0.70
6-1-14	11.52	9	0.59
6-2-1	11.46	11	0.73
6-2-2	16.41	27	0.61
6-2-3	17.97	41	0.71
6-2-4	15.53	25	0.67
6-2-5	11.17	10	0.72
6-2-6	16.19	27	0.64
6-2-7	10.95	11	0.84
6-2-8	16.84	31	0.65
6-2-9	10.02	7	0.70
6-2-10	12.10	13	0.73
6-2-11	15.60	26	0.68
6-2-12	14.28	19	0.65
6-2-13	16.68	32	0.69
6-2-14	11.71	11	0.69
6-3-1	9.23	5	0.64
6-3-2	10.26	11	1.02
6-3-3	16.33	30	0.69
6-3-4	16.01	25	0.61
6-3-5	13.52	19	0.77
6-3-6	13.52	20	0.81

6-3-7	11.67	15	0.94
6-3-8	12.22	13	0.71
6-3-9	16.69	27	0.58
6-3-10	11.25	9	0.63
6-3-11	10.84	11	0.86
6-3-12	12.60	14	0.70
6-3-13	13.87	15	0.56
6-3-14	10.42	8	0.71

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: EE1+NP100

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
7-1-1	13.35	14	0.59
7-1-2	15.61	22	0.58
7-1-3	12.52	13	0.66
7-1-4	12.74	13	0.63
7-1-5	11.41	8	0.54
7-1-6	14.84	21	0.64
7-1-7	11.20	11	0.78
7-1-8	18.35	40	0.65
7-1-9	13.92	15	0.56
7-1-10	16.42	26	0.59
7-1-11	12.97	14	0.64
7-1-12	15.22	20	0.57
7-1-13	15.22	21	0.60
7-1-14	12.22	11	0.60
7-2-1	10.88	9	0.70
7-2-2	12.76	15	0.72
7-2-3	13.58	17	0.68
7-2-4	10.08	9	0.88
7-2-5	9.21	7	0.90
7-2-6	11.35	10	0.68
7-2-7	12.52	13	0.66
7-2-8	14.47	20	0.66
7-2-9	11.23	11	0.78
7-2-10	9.18	4	0.52
7-2-11	11.87	13	0.78
7-2-12	10.44	8	0.70
7-2-13	15.21	23	0.65
7-2-14	8.93	5	0.70
7-3-1	11.49	11	0.73
7-3-2	8.95	5	0.70
7-3-3	10.13	8	0.77
7-3-4	14.21	18	0.63
7-3-5	9.21	5	0.64

7-3-6	13.2	15	0.65
7-3-7	9.59	6	0.68
7-3-8	14.3	17	0.58
7-3-9	10.64	9	0.75
7-3-10	12.8	15	0.72
7-3-11	9.25	5	0.63
7-3-12	10.55	10	0.85
7-3-13	9.92	7	0.72
7-3-14	14.03	22	0.80

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: EE10+NP10

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
8-1-1	11.89	13	0.77
8-1-2	9.34	6	0.74
8-1-3	10.20	7	0.66
8-1-4	10.02	7	0.70
8-1-5	9.03	6	0.81
8-1-6	10.54	11	0.94
8-1-7	9.11	5	0.66
8-1-8	9.35	7	0.86
8-1-9	8.65	6	0.93
8-1-10	8.65	5	0.77
8-1-11	8.49	4	0.65
8-1-12	7.92	4	0.81
8-1-13	7.28	2	0.52
8-1-14	9.19	5	0.64
8-1-15	8.03	5	0.97
8-2-1	7.86	4	0.82
8-2-2	7.28	3	0.78
8-2-3	7.85	5	1.03
8-2-4	9.13	7	0.92
8-2-5	9.60	9	1.02
8-2-6	12.04	15	0.86
8-2-7	9.96	9	0.91
8-2-8	9.40	7	0.84
8-2-9	11.14	11	0.80
8-2-10	9.12	6	0.79
8-2-11	10.12	11	1.06
8-2-12	10.32	11	1.00
8-2-13	9.05	5	0.67
8-2-14	11.11	12	0.88
8-2-15	10.12	9	0.87
8-3-1	13.51	21	0.85
8-3-2	8.75	6	0.90

8-3-3	8.75	5	0.75
8-3-4	11.48	13	0.86
8-3-5	8.97	5	0.69
8-3-6	9.82	9	0.95
8-3-7	12.92	16	0.74
8-3-8	8.00	8	1.56
8-3-9	10.54	9	0.77
8-3-10	8.66	4	0.62
8-3-11	9.19	8	1.03
8-3-12	9.19	6	0.77
8-3-13	9.51	9	1.05
8-3-14	8.85	6	0.87

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

Treatment: EE10+NP100

Sample #	Length (mm)	Weight (mg)	Condition Factor[1]
9-1-1	12.06	13	0.74
9-1-2	9.04	5	0.68
9-1-3	9.04	5	0.68
9-1-4	11.07	8	0.59
9-1-5	9.78	7	0.75
9-1-6	9.78	8	0.86
9-1-7	10.8	9	0.71
9-1-8	11.18	9	0.64
9-1-9	9.71	5	0.55
9-1-10	11.39	12	0.81
9-1-11	9.42	5	0.60
9-1-12	10.56	8	0.68
9-1-13	12.39	13	0.68
9-1-14	9.30	6	0.75
9-2-1	8.30	5	0.87
9-2-2	9.94	9	0.92
9-2-3	9.29	6	0.75
9-2-4	10.93	9	0.69
9-2-5	10.38	8	0.72
9-2-6	11.15	11	0.79
9-2-7	8.43	5	0.83
9-2-8	12.05	15	0.86
9-2-9	9.22	5	0.64
9-2-10	11.64	12	0.76
9-2-11	10.31	10	0.91
9-2-12	9.36	7	0.85
9-2-13	8.16	4	0.74
9-2-14	9.89	8	0.83
9-3-1	10.42	12	1.06

9-3-2	9.39	7	0.85
9-3-3	8.29	4	0.70
9-3-4	10.26	8	0.74
9-3-5	9.84	7	0.73
9-3-6	9.21	4	0.51
9-3-7	10.21	8	0.75
9-3-8	9.63	8	0.90
9-3-9	9.63	5	0.56
9-3-10	9.89	7	0.72
9-3-11	9.87	9	0.94
9-3-12	8.70	6	0.91
9-3-13	10.63	8	0.67
9-3-14	8.96	7	0.97

[1]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

APPENDIX D
SIXTY DAYS POST-HATCH GONADAL STAGING DATA

Blind #	Sample #	Sex	Oo	PreV	% Oo	% PreV	Sg	Sc	St	%Sg	%Sc	%St
44	1-1-07	F	34	1	97.14	2.86						
47	1-1-08	F	54	30	64.29	35.71						
50	1-1-09	F	42	39	51.85	48.15						
82	1-1-10	F	35	4	89.74	10.26						
61	1-1-11	F	71	141	33.49	66.51						
51	1-1-12	F	37	183	16.82	83.18						
58	1-1-14	F	77	117	39.69	60.31						
90	1-2-08	F	9	3	75.00	25.00						
70	1-2-09	F	40	29	57.97	42.03						
77	1-2-10	F	23	69	25.00	75.00						
81	1-2-11	F	15	2	88.24	11.76						
29	1-2-13	F	68	7	90.67	9.33						
9	1-2-14	M					20	7	0	74.10	26.00	0
75	1-3-03	F	38	106	26.39	73.61						
48	1-3-04	F	55	3	94.83	5.17						
83	1-3-05	F	74	78	48.68	51.32						
33	1-3-06	F	17	12	58.62	41.38						
91	1-3-07	F	53	23	69.74	30.26						
35	3-1-08	F	167	346	32.55	67.45						
76	3-1-10	M					28	3	0	90.30	9.70	0
7	3-1-12	F	145	179	44.75	55.25						
31	3-1-14	F	308	104	74.76	25.24						
64	3-2-09	M					57	5	0	91.90	8.10	0
102	3-2-10	M					42	59	6	39.30	55	5.61
100	3-2-12	M					94	13	1	87.00	12.00	0.93
79	3-2-13	F	122	319	27.66	72.34						
59	3-2-14	F	113	375	23.16	76.84						
2	3-3-01	F	240	45	84.21	15.79						
20	3-3-02	F	88	256	25.58	74.42						
60	3-3-03	F	69	218	24.04	75.96						
16	3-3-04	F	54	292	15.61	84.39						
28	3-3-05	F	69	236	22.62	77.38						
86	3-3-06	M					108	157	106	29.10	42.00	28.60
88	3-3-07	F	86	395	17.88	82.12						

66	4-1-08	F	35	140	20.00	80.00						
72	4-1-09	F	13	24	35.14	64.86						
71	4-1-10	F	21	85	19.81	80.19						
57	4-1-11	F	51	60	45.95	54.05						
13	4-1-14	F	94	19	83.19	16.81						
74	4-2-10	F	36	17	67.92	32.08						
68	4-2-12	F	25	7	78.13	21.88						
36	4-2-13	F	23	4	85.19	14.81						
62	4-2-14	F	106	8	92.98	7.02						
1	4-3-01	F	64	22	74.42	25.58						
52	4-3-02	F	41	2	95.35	4.65						
53	4-3-03	F	37	76	32.74	67.26						
22	4-3-04	F	78	147	34.67	65.33						
25	4-3-06	F	243	58	80.73	19.27						
99	4-3-07	F	98	7	93.33	6.67						
34	4-3-11	F	98	152	39.20	60.80						
10	5-1-09	M					112	35	7	72.70	23.00	4.55
92	5-1-10	M					146	14	3	89.60	8.60	1.84
12	5-1-11	M					86	21	10	73.50	18.00	8.55
67	5-1-12	F	121	607	16.62	83.38						
5	5-1-13	F	121	326	27.07	72.93						
48	5-1-14	F	88	431	16.96	83.04						
94	5-2-01	M					66	50	49	40.00	30.00	29.70
49	5-2-08	F	38	240	13.67	86.33						
65	5-2-10	M					49	9	0	84.50	16.00	0
38	5-2-11	F	56	267	17.34	82.66						
54	5-2-12	M					65	8	5	83.30	10.00	6.41
96	5-3-03	M					32	41	35	29.60	38.00	32.40
23	5-3-05	M					72	77	84	30.90	33.00	36.10
14	5-3-06	M					22	69	48	15.80	50.00	34.50
42	6-1-01	F	39	175	18.22	81.78						
55	6-1-08	F	31	12	72.09	27.91						
63	6-1-09	F	55	8	87.30	12.70						
80	6-1-10	F	69	50	57.98	42.02						
45	6-1-12	F	88	15	85.44	14.56						
78	6-2-09	F	48	49	49.48	50.52						
37	6-2-12	F	16	12	57.14	42.86						
40	6-2-13	F	41	150	21.47	78.53						
41	6-2-14	F	40	24	62.50	37.50						
4	6-3-01	F	31	63	32.98	67.02						
85	6-3-02	F	14	6	70.00	30.00						
97	6-3-03	F	49	4	92.45	7.55						
26	6-3-05	F	61	57	51.69	48.31						
27	6-3-07	F	74	126	37.00	63.00						
8	7-1-12	M					49	4	5	0.84	6.9	8.62
73	7-1-13	F	85	57	59.86	40.14						

43	7-1-14	F	46	17	73.02	26.98
32	7-2-08	F	21	15	58.33	41.67
30	7-2-10	F	8	20	28.57	71.43
87	7-2-13	F	45	15	75.00	25.00
98	7-2-14	F	24	5	82.76	17.24
84	7-3-01	F	27	45	37.50	62.50
39	7-3-02	F	66	167	28.33	71.67
21	7-3-04	F	60	51	54.05	45.95
6	7-3-05	F	82	48	63.08	36.92
15	7-3-06	F	51	96	34.69	65.31
24	7-3-07	F	29	2	93.55	6.45

Oo: oogonia
% Oo: percent oogonia
PreV: previtellogenic
% PreV: percent previtellogenic
Sg: spermatogonia
% Sg: percent spermatogonia
Sc: primary or secondary spermatocyte
% Sc: percent primary or secondary spermatocyte
St: spermatid
% St: percent spermatid

APPENDIX E
ADULT ZEBRAFISH (240 DPH) DATA

Treatment: Control

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
5-01	M	38.6	395	0.69	0.710
5-02	M	36.5	401	0.83	1.097
5-03	M	37.1	387	0.76	1.187
5-04	M	37.4	363	0.70	1.046
5-05	M	44.3	811	0.94	2.676
5-06	M	38.6	442	0.77	0.837
5-07	F	47.0	1099	1.06	10.332
5-08	M	45.4	861	0.92	2.254
5-09	M	39.4	475	0.78	0.947
5-10	F	44.1	924	1.08	13.040
5-11	F	45.5	1199	1.28	18.562
5-12	F	48.1	1172	1.05	10.920
5-13	F	45.2	1253	1.36	32.184
5-14	F	46.8	1200	1.17	16.686
5-15	F	45.6	1063	1.12	11.725
5-16	F	45.6	1346	1.42	19.100
5-17	M	40.2	558	0.86	1.470
5-18	M	36.1	486	1.03	1.665
5-19	M	35.8	386	0.84	0.751
5-20	M	43.6	836	1.01	1.818
5-21	M	36.2	491	1.04	1.325
5-22	M	44.0	868	1.02	1.487
5-23	M	35.0	352	0.82	1.081
5-24	M	37.0	435	0.86	1.128
5-25	F	34.6	284	0.68	18.035
5-26	M	37.6	402	0.76	1.395
5-27	F	35.4	331	0.75	20.182
5-28	M	39.3	534	0.88	0.881
5-29	F	33.0	307	0.85	17.634
5-30	F	35.7	382	0.84	13.799
5-31	M	41.2	703	1.00	1.566
5-32	F	33.8	328	0.85	26.821
5-33	F	34.5	364	0.88	20.561

5-34	F	37.0	395	0.78	17.046
5-35	M	31.8	308	0.96	0.845
5-36	M	37.4	458	0.87	1.047
5-37	M	38.6	467	0.81	1.264
5-38	F	37.4	435	0.83	21.905
5-39	M	41.5	603	0.85	0.995
5-40	F	38.0	401	0.73	27.001
5-41	M	38.3	444	0.79	0.789
5-42	M	38.8	471	0.81	1.402
5-43	M	38.5	434	0.76	1.013
5-44	M	38.4	485	0.86	0.763
5-45	M	40.1	572	0.89	1.136
5-46	M	40.2	504	0.78	1.032
5-47	M	38.5	477	0.83	1.490
5-48	F	41.0	698	1.01	22.841
5-49	F	42.2	934	1.24	12.236
5-50	F	37.4	544	1.04	23.217
5-51	F	39.8	853	1.36	17.713
5-52	M	40.5	595	0.89	0.891
5-53	F	44.6	935	1.06	14.202
5-54	F	38.5	688	1.21	17.212
5-55	F	40.4	626	0.95	16.621
5-56	F	39.9	842	1.33	13.507
5-57	F	41.2	820	1.17	13.218
5-58	M	39.4	517	0.85	1.470
5-59	M	41.2	599	0.85	1.368
5-60	M	40.8	558	0.82	1.542
5-61	M	39.0	588	0.99	1.650
5-62	M	40.2	586	0.90	1.724
5-63	M	38.9	549	0.94	1.238

[1]Sex: M = male, F = female

[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100

Treatment: NP10

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
3-01	F	47.5	1086	1.01	12.923
3-02	F	46.7	1091	1.07	19.388
3-03	F	45.3	1119	1.20	16.537
3-04	F	43.6	877	1.06	15.733
3-05	F	49.3	1523	1.27	19.048
3-06	F	44.6	1205	1.36	28.983
3-07	M	45.4	802	0.86	1.358
3-08	M	42.2	655	0.87	0.901

3-09	M	42.6	697	0.90	1.563
3-10	F	43.0	834	1.05	12.478
3-11	F	43.1	932	1.16	18.143
3-12	F	41.3	818	1.17	16.789
3-13	M	41.6	657	0.91	1.477
3-14	M	41.4	688	0.97	1.569
3-15	M	34.1	355	0.89	0.761
3-16	M	39.3	514	0.85	1.303
3-17	M	42.6	801	1.04	1.910
3-18	M	39.9	588	0.92	2.126
3-19	M	40.9	798	1.17	1.717
3-20	M	35.3	347	0.79	0.663
3-21	M	42.5	714	0.93	1.429
3-22	F	41.0	900	1.30	42.946
3-23	M	35.9	406	0.88	0.591
3-24	M	40.1	653	1.02	1.271
3-25	F	32.3	379	1.13	14.828
3-26	F	35.8	476	1.04	17.489
3-27	M	38.9	432	0.74	0.486
3-28	M	36.2	326	0.69	0.582
3-29	M	38.3	471	0.84	0.743
3-30	F	32.1	270	0.82	11.428
3-31	F	36.3	426	0.89	9.195
3-32	M	38.1	392	0.71	0.740
3-33	M	35.2	325	0.75	0.277
3-34	F	38.4	491	0.87	9.037
3-35	M	41.1	607	0.88	1.614
3-36	M	37.3	411	0.79	0.657
3-37	M	35.3	334	0.76	0.658
3-38	M	40.1	505	0.78	0.931
3-39	F	34.1	351	0.89	6.530
3-40	M	35.7	307	0.68	0.325
3-41	M	41.1	615	0.89	1.236
3-42	F	35.7	390	0.86	12.510
3-43	F	37.3	529	1.02	12.077
3-44	F	45.7	950	0.99	11.360
3-45	M	36.7	368	0.75	0.979
3-46	F	37.0	398	0.79	2.865
3-47	M	36.2	332	0.70	0.663
3-48	M	37.4	408	0.78	0.981
3-49	F	33.9	367	0.94	11.959
3-50	F	34.9	390	0.92	11.707
3-51	F	35.5	374	0.84	9.749
3-52	F	31.6	285	0.90	16.052

[1]Sex: M = male, F = female

[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100

Treatment: NP100

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
4-01	M	39.9	601	0.95	1.231
4-02	M	37.1	447	0.88	0.694
4-03	M	38.8	626	1.07	1.678
4-04	M	39.4	543	0.89	1.252
4-05	M	39.6	570	0.92	1.297
4-06	F	42.5	1045	1.36	12.758
4-07	M	35.6	466	1.04	0.943
4-08	M	39.5	588	0.95	1.530
4-09	M	37.9	574	1.05	1.098
4-10	M	41.8	759	1.04	1.160
4-11	F	40.0	854	1.34	18.954
4-12	F	45.9	1161	1.20	15.580
4-13	M	37.6	451	0.85	1.331
4-14	F	44.7	857	0.96	11.520
4-15	M	38.9	529	0.90	1.418
4-16	M	37.9	507	0.93	0.928
4-17	M	37.9	478	0.88	1.568
4-18	F	38.9	539	0.92	13.198
4-19	M	38.0	580	1.06	0.966
4-20	F	38.1	533	0.96	3.023
4-21	F	40.9	795	1.16	11.789
4-22	M	36.2	549	1.16	1.238
4-23	M	38.1	591	1.07	1.150
4-24	M	40.5	591	0.89	1.574
4-25	M	40.3	643	0.98	1.338
4-26	M	38.4	610	1.08	1.477
4-27	M	37.6	471	0.89	1.358
4-28	M	38.9	672	1.15	2.038
4-29	M	40.4	755	1.15	1.377
4-30	M	40.0	611	0.95	1.079
4-31	F	41.7	742	1.02	10.371
4-32	M	38.0	536	0.98	1.175
4-33	M	37.7	549	1.02	1.549

[1]Sex: M = male, F = female
[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000
[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100

Treatment: EE1

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition	GSI[3]

				Factor[2]	
1-01	M	42.4	765	1.00	N/A[4]
1-02	M	45.5	745	0.79	N/A[4]
1-03	F	45.1	1080	1.18	25.278
1-04	F	43.4	1045	1.28	20.861
1-05	F	45.9	1445	1.49	27.820
1-06	F	47.5	1205	1.12	18.340
1-07	M	38.7	473	0.82	N/A[4]
1-08	M	44.1	812	0.94	0.985
1-09	F	43.9	921	1.09	9.989
1-10	F	46.3	1096	1.10	16.971
1-11	F	46.1	1182	1.21	15.821
1-12	F	48.3	1262	1.12	19.572
1-13	M	38.0	439	0.80	1.139
1-14	M	42.5	826	1.08	0.969
1-15	F	38.4	692	1.22	20.376
1-16	M	37.5	510	0.97	1.176
1-17	M	36.5	424	0.87	0.943
1-18	M	36.0	381	0.82	0.787
1-19	M	39.5	510	0.83	1.373
1-20	F	39.8	616	0.98	14.935
1-21	F	41.7	604	0.83	12.583
1-22	M	35.3	393	0.90	1.018
1-23	M	35.8	289	0.63	0.692
1-24	M	38.9	473	0.80	0.846
1-25	M	37.8	436	0.81	1.147
1-26	F	34.7	329	0.78	12.158
1-27	M	40.2	572	0.88	0.874
1-28	M	39.5	457	0.74	0.656
1-29	M	38.2	436	0.78	0.917
1-30	M	39.2	443	0.74	1.354
1-31	M	40.2	484	0.74	1.033
1-32	M	35.8	417	0.91	0.959
1-33	M	38.1	433	0.78	0.693
1-34	M	37.8	443	0.82	0.677
1-35	M	36.8	414	0.83	1.932
1-36	M	38.6	500	0.87	0.600
1-37	F	39.3	642	1.06	11.215
1-38	M	37.1	465	0.91	1.720
1-39	F	37.4	672	1.28	22.619
1-40	F	35.2	361	0.83	8.587
1-41	M	39.0	513	0.87	1.365
1-42	F	37.1	475	0.93	12.211
1-43	M	40.1	546	0.85	1.648
1-44	F	36.2	503	1.06	21.471
1-45	F	35.2	279	0.64	3.226

1-46	M	36.0	377	0.81	0.796
1-47	M	36.8	462	0.92	1.082
1-48	F	30.3	216	0.78	10.185
1-49	F	41.3	632	0.90	10.443
1-50	M	37.6	454	0.86	0.881
1-51	M	34.6	380	0.92	0.789
1-52	M	37.9	475	0.88	1.684
1-53	M	36.2	407	0.86	0.983
1-54	F	37.5	594	1.13	15.657
1-55	M	40.3	610	0.93	1.311
1-56	F	38.8	486	0.84	12.346
1-57	F	33.9	364	0.93	22.802
1-58	M	35.4	440	0.99	1.591
1-59	F	39.4	583	0.96	11.492
1-60	M	38.1	433	0.79	1.155
1-61	M	38.9	493	0.84	1.420
1-62	M	39.4	505	0.82	0.990
1-63	M	37.2	390	0.76	0.769
1-64	M	38.4	499	0.88	0.802
1-65	M	38.1	554	1.00	1.083
1-66	F	40.2	733	1.13	20.737
1-67	F	40.5	740	1.11	23.919

[1]Sex: M = male, F = female
[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000
[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100
[4]These fish were the first three male fish dissected. Due to inexperience, testes were not properly identified and saved.

Treatment: EE10

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
2-01	M	38.2	535	0.96	1.308
2-02	M	39.7	568	0.91	1.056
2-03	M	41.1	609	0.88	0.985
2-04	M	38.9	584	0.99	1.027
2-05	F	39.8	690	1.10	10.00
2-06	M	33.6	350	0.92	0.857
2-07	M	40.8	723	1.06	1.521
2-08	M	40.3	607	0.93	1.153
2-09	M	41.1	684	0.99	0.877
2-10	M	41.6	674	0.94	1.335
2-11	M	41.6	696	0.97	1.293
2-12	M	38.9	556	0.95	1.799
2-13	M	39.8	609	0.96	0.821
2-14	M	35.0	408	0.95	0.980

2-15	M	33.8	286	0.74	0.699
2-16	F	31.4	236	0.76	4.661
2-17	M	33.6	344	0.91	0.581
2-18	M	35.7	299	0.66	1.003
2-19	M	39.1	398	0.67	N/A[4]
2-20	M	32.0	271	0.83	0.738
2-21	M	35.3	368	0.84	0.272
2-22	M	32.3	253	0.75	0.395
2-23	M	34.0	280	0.72	0.714
2-24	M	40.3	573	0.88	1.222
2-25	M	31.7	250	0.78	0.400
2-26	M	34.3	356	0.88	0.562
2-27	M	38.4	555	0.98	2.162
2-28	M	36.9	340	0.68	0.294
2-29	M	40.8	580	0.86	0.517
2-30	M	30.6	234	0.82	0.427
2-31	M	34.1	323	0.82	0.310
2-32	M	40.3	372	0.57	0.269
2-33	M	35.2	308	0.71	0.325
2-34	F	29.2	222	0.89	9.910
2-35	M	36.1	438	0.93	0.685
2-36	M	36.6	316	0.64	0.316
2-37	M	31.1	209	0.70	0.718
2-38	M	35.6	322	0.72	0.373
2-39	M	37.4	372	0.71	0.269
2-40	M	31.6	238	0.75	0.462
2-41	M	37.2	239	0.46	N/A[4]
2-42	M	35.0	353	0.83	0.850
2-43	M	33.2	281	0.77	0.640
2-44	M	32.0	329	1.00	0.425
2-45	M	36.4	409	0.84	0.734
2-46	M	35.0	333	0.77	0.691
2-47	M	39.7	451	0.72	0.864
2-48	M	34.6	295	0.71	0.949
2-49	M	35.9	336	0.73	1.222
2-50	M	37.5	417	0.79	1.342
2-51	M	36.7	362	0.73	0.800
2-52	M	34.8	326	0.78	0.522
2-53	M	34.9	349	0.82	0.918
2-54	M	42.1	801	1.07	1.785
2-55	M	46.2	1041	1.05	1.470
2-56	M	47.2	1018	0.97	1.846
2-57	M	44.0	839	0.99	1.156
2-58	M	44.0	780	0.92	1.192
2-59	M	43.4	742	0.91	1.118
2-60	M	46.0	1110	1.14	1.495

2-61	M	47.8	1066	0.98	2.045
2-62	F	50.5	1974	1.54	28.579
2-63	F	49.3	1794	1.50	30.308
2-64	M	47.9	1133	1.03	2.532
2-65	M	45.0	944	1.04	1.980

[1]Sex: M = male, F = female
[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000
[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100
[4]The testes of these fish were too small to be weighed and preserved.

Treatment: EE1+NP10

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
6-01	M	38.7	511	0.89	0.821
6-02	M	42.6	640	0.83	1.328
6-03	M	39.4	543	0.89	1.272
6-04	M	38.1	478	0.87	1.045
6-05	M	36.8	374	0.75	1.257
6-06	F	43.1	693	0.87	11.652
6-07	M	40.1	551	0.86	1.289
6-08	F	39.3	568	0.93	12.052
6-09	M	39.9	535	0.84	1.120
6-10	M	40.1	605	0.94	2.298
6-11	M	37.4	558	1.07	1.094
6-12	M	37.2	473	0.92	1.439
6-13	M	39.0	604	1.02	1.274
6-14	M	39.8	578	0.92	1.870
6-15	M	39.9	592	0.93	1.014
6-16	M	39.6	578	0.93	1.418
6-17	M	39.2	546	0.91	1.704
6-18	M	38.3	569	1.01	1.494
6-19	M	39.4	516	0.84	1.259
6-20	M	38.9	521	0.89	2.072
6-21	M	40.0	534	0.84	1.460
6-22	F	41.3	727	1.03	18.422
6-23	M	38.9	556	0.94	1.078
6-24	M	40.1	607	0.94	1.911
6-25	M	37.5	497	0.94	1.608
6-26	F	44.1	1103	1.29	26.605
6-27	M	37.7	524	0.97	1.814
6-28	F	42.4	1129	1.48	20.417
6-29	M	40.4	680	1.03	1.353
6-30	M	39.6	568	0.92	1.321
6-31	M	39.0	535	0.90	1.215
6-32	M	33.0	288	0.80	0.382

6-33	F	44.5	842	0.96	9.529
6-34	F	44.4	1163	1.32	23.163
6-35	F	42.1	864	1.16	23.586
6-36	F	45.3	1158	1.25	26.786
6-37	F	39.9	754	1.19	19.548
6-38	M	43.1	855	1.07	1.766
6-39	F	43.0	888	1.12	15.713
6-40[4]	F	N/A	N/A	N/A	N/A

[1]Sex: M = male, F = female
[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000
[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100
[4]One carcass with partial ovary was found during daily check-up. No available weight and length information as the carcass was partially decomposed when discovered.

Treatment: EE1+NP100

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
7-01	F	39.1	633	1.06	22.671
7-02	M	39.5	565	0.92	1.150
7-03	M	36.2	449	0.95	1.090
7-04	M	37.5	501	0.95	1.476
7-05	M	39.6	538	0.86	0.781
7-06	M	37.9	570	1.05	1.826
7-07	M	39.3	519	0.86	1.369
7-08	M	40.1	637	0.99	1.382
7-09	M	39.2	572	0.95	1.504
7-10	F	40.0	725	1.14	21.390
7-11	M	37.4	482	0.92	1.059
7-12	M	36.9	453	0.90	1.169
7-13	M	38.8	474	0.81	0.950
7-14	M	36.3	466	0.97	1.587
7-15	M	38.5	486	0.85	1.215
7-16	F	42.9	841	1.07	12.724
7-17	M	39.4	490	0.80	1.491
7-18	M	39.4	599	0.98	1.702
7-19	M	38.5	531	0.93	1.243
7-20	M	36.9	473	0.94	0.909
7-21	M	37.7	457	0.86	1.509
7-22	M	38.8	630	1.08	1.443
7-23	F	44.8	972	1.08	19.449
7-24	F	42.4	930	1.22	10.823
7-25	M	39.1	617	1.04	1.992
7-26	F	41.1	838	1.21	24.766
7-27	F	38.8	748	1.29	13.444
7-28	M	40.7	662	0.98	1.707

7-29	F	39.4	930	1.52	23.621
7-30	F	44.1	1131	1.32	25.277
7-31	F	41.8	910	1.25	14.228
7-32	F	41.7	765	1.05	14.828
7-33	F	41.1	758	1.09	11.124
7-34	M	38.7	590	1.02	1.169
7-35	M	39.1	491	0.82	1.139
7-36	M	38.6	503	0.87	1.015
7-37	M	39.9	683	1.07	1.143
7-38	M	39.5	552	0.90	1.577
7-39	M	38.5	548	0.96	1.260
7-40[4]	F	N/A	N/A	N/A	N/A
7-41[4]	F	N/A	N/A	N/A	N/A
7-42[4]	F	N/A	N/A	N/A	N/A

[1]Sex: M = male, F = female
[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000
[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100
[4]Three carcasses with partial ovary were found on three separate occasions during daily check-up. No available weight and length information as the carcass was partially decomposed when discovered.

Treatment: EE10+NP10

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
8-01	M	43.6	740	0.89	1.744
8-02	M	41.4	696	0.98	1.637
8-03	M	44.4	914	1.05	1.389
8-04	M	40.9	662	0.97	1.857
8-05	M	42.9	699	0.89	1.802
8-06	M	39.0	643	1.08	1.337
8-07	M	41.2	686	0.98	1.471
8-08	M	41.8	681	0.93	2.276
8-09	M	41.6	675	0.94	1.970
8-10	M	39.9	617	0.97	1.750
8-11	M	39.1	517	0.87	1.045
8-12	M	44.0	774	0.91	1.758
8-13	M	41.2	697	1.00	2.153
8-14	M	39.9	540	0.85	1.501
8-15	M	43.0	729	0.92	1.564
8-16	M	40.0	656	1.02	1.250
8-17	M	36.7	449	0.91	0.735
8-18	M	37.3	495	0.96	1.374
8-19	F	38.7	861	1.49	22.033
8-20	M	38.4	499	0.88	1.503
8-21	M	37.8	583	1.08	1.217

8-22	M	36.6	327	0.67	0.642
8-23	M	44.3	785	0.90	1.604
8-24	M	38.8	527	0.90	1.216
8-25	M	41.4	554	0.78	1.660
8-26	M	40.0	558	0.88	1.362
8-27	M	36.4	463	0.96	0.973
8-28	M	43.1	740	0.92	2.217
8-29	M	35.7	355	0.78	0.733
8-30	M	36.9	522	1.04	1.705
8-31	M	38.8	486	0.83	1.461
8-32	M	37.6	416	0.78	1.348
8-33	M	40.7	71	0.11	12.669
8-34	M	38.2	514	0.92	0.818
8-35	M	42.4	662	0.87	1.708
8-36	M	40.9	599	0.88	1.253
8-37	M	39.5	484	0.79	1.095
8-38	M	40.4	651	0.99	1.629
8-39	M	41.7	595	0.82	1.562
8-40	M	39.2	599	0.99	1.402
8-41	M	37.1	433	0.85	1.108
8-42	M	39.2	532	0.88	1.578
8-43	M	34.7	323	0.77	1.175
8-44	M	42.5	598	0.78	1.271
8-45	M	39.9	642	1.01	1.309
8-46	M	35.4	371	0.83	0.782
8-47	M	37.9	473	0.87	0.909
8-48	M	40.2	564	0.87	1.703
8-49	M	40.5	765	1.15	0.484

[1]Sex: M = male, F = female

[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100

Treatment: EE10+NP100

Sample #	Sex[1]	Length (mm)	Weight (mg)	Condition Factor[2]	GSI[3]
9-01	F	43.8	1349	1.60	25.400
9-02	F	47.0	1186	1.14	16.061
9-03	M	44.2	782	0.91	2.047
9-04	M	42.0	864	1.16	3.205
9-05	M	41.6	813	1.13	2.214
9-06	M	43.4	822	1.00	1.802
9-07	M	44.6	820	0.93	1.769
9-08	M	42.7	791	1.02	1.706
9-09	M	43.6	790	0.95	2.191
9-10	M	44.2	901	1.05	2.008

9-11	F	39.6	675	1.09	9.242
9-12	F	36.2	497	1.05	12.155
9-13	M	39.1	445	0.75	1.192
9-14	M	37.0	378	0.75	1.163
9-15	M	36.3	411	0.86	0.779
9-16	M	36.8	416	0.84	1.082
9-17	F	36.5	391	0.81	12.832
9-18	M	39.0	460	0.78	0.717
9-19	M	38.5	463	0.81	0.928
9-20	M	34.8	322	0.76	0.404
9-21	M	35.2	488	1.12	1.025
9-22	M	36.7	405	0.82	0.939
9-23	M	36.3	478	1.00	0.774
9-24	M	36.7	379	0.77	0.792
9-25	M	36.5	395	0.81	0.659
9-26	M	35.8	380	0.83	0.711
9-27	M	34.8	400	0.95	0.475
9-28	M	33.0	298	0.83	0.571
9-29	M	37.9	443	0.81	1.220
9-30	M	36.1	308	0.66	0.389
9-31	M	39.1	528	0.88	1.251
9-32	M	38.3	522	0.93	1.706
9-33	M	38.1	465	0.84	1.462
9-34	M	37.9	446	0.82	1.121
9-35	M	40.0	561	0.88	1.711
9-36	M	37.5	427	0.81	1.102
9-37	M	39.3	481	0.79	0.770
9-38	M	34.4	264	0.65	0.834
9-39	M	37.5	439	0.83	0.956
9-40	M	38.2	434	0.78	0.691
9-41	M	37.7	435	0.81	1.633
9-42	M	35.4	341	0.77	1.495
9-43	M	39.2	505	0.84	1.168
9-44	M	40.4	492	0.75	0.786
9-45	M	40.1	484	0.75	1.012
9-46	M	39.6	554	0.89	0.938
9-47	M	38.6	420	0.73	0.810
9-48	M	38.1	457	0.83	1.072
9-49	M	40.4	557	0.84	1.384
9-50	M	36.6	374	0.76	0.669
9-51	M	33.8	289	0.75	0.762
9-52	M	34.5	314	0.77	0.638
9-53	M	34.4	317	0.78	1.105
9-54	M	34.8	335	0.79	0.984
9-55	M	39.4	470	0.77	1.298
9-56	M	36.2	425	0.90	1.084

9-57	M	32.8	310	0.88	0.580
9-58	M	33.9	320	0.82	0.781
9-59	M	35.4	359	0.81	0.445
9-60	M	35.1	448	1.03	1.383
9-61	M	31.4	239	0.77	0.836
9-62	M	31.2	230	0.75	0.436
9-63	M	36.1	347	0.74	0.806
9-64	M	39.0	516	0.87	1.163
9-65	M	37.5	432	0.82	0.625
9-66	M	35.0	406	0.95	0.763
9-67	M	35.4	421	0.95	1.759
9-68	M	35.6	429	0.95	0.886
9-69	M	37.4	425	0.81	0.705
9-70	M	36.7	411	0.83	0.681
9-71	M	34.0	341	0.87	0.880
9-72	M	34.4	351	0.86	0.855

[1]Sex: M = male, F = female

[2]Condition Factor = [(body weight (g)/length (mm)^3] × 100,000

[3]GSI (Gonadosomatic index) = (gonad weight (mg)) / (body weight (g)) × 100

APPENDIX F
ADULT REPRODUCTIVE STUDY DATA

Treatment: Control

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	133	109	82.0	108	99.1	84	77.1	77.8
2	107	100	93.5	100	100.0	95	95.0	95.0
3	35	32	91.4	30	93.8	21	65.6	70.0
4	280	152	54.3	151	99.3	144	94.7	95.4
5	140	0	0.0	0	N/A	N/A	N/A	N/A
6	166	133	80.1	74	55.6	57	42.9	77.0
7	153	130	85.0	130	100.0	122	93.8	93.8
8	34	33	97.1	33	100.0	33	100.0	100.0
9	52	43	82.7	4	9.3	4	9.3	100.0
10	98	90	91.8	90	100.0	85	94.4	94.4
11	740	631	85.3	360	57.1	342	54.2	95.0
12	102	76	74.5	75	98.7	70	92.1	93.3
13	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14	496	389	78.4	342	87.9	341	87.7	99.7
15	127	99	78.0	94	94.9	85	85.9	90.4
16	200	156	78.0	104	66.7	84	53.8	80.8
17	224	167	74.6	137	82.0	126	75.4	92.0
18	317	290	91.5	238	82.1	224	77.2	94.1
19	251	188	74.9	140	74.5	128	68.1	91.4
20	328	289	88.1	249	86.2	224	77.5	90.0
21	246	197	80.1	157	79.7	145	73.6	92.4
22	212	167	78.8	84	50.3	84	50.3	100.0
23	298	238	79.9	237	99.6	215	90.3	90.7
24	291	262	90.0	152	58.0	150	57.3	98.7
25	169	145	85.8	115	79.3	95	65.5	82.6
26	136	85	62.5	76	89.4	56	65.9	73.7
27	150	120	80.0	115	95.8	105	87.5	91.3
28	167	142	85.0	94	66.2	88	62.0	93.6
29	227	169	74.4	156	92.3	132	78.1	84.6
30	160	112	70.0	106	94.6	95	84.8	89.6
31	246	230	93.5	118	51.3	95	41.3	80.5
32	515	468	90.9	374	79.9	372	79.5	99.5
33	261	219	83.9	205	93.6	184	84.0	89.8

34	9	6	66.7	6	100.0	5	83.3	83.3
35	357	324	90.8	285	88.0	256	79.0	89.8
36	561	478	85.2	462	96.7	405	84.7	87.7
37	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
38	934	711	76.1	705	99.2	524	73.7	74.3
39	387	367	94.8	315	85.8	297	80.9	94.3
40	364	250	68.7	247	98.8	244	97.6	98.8
41	310	277	89.4	275	99.3	214	77.3	77.8
42	47	40	85.1	40	100.0	40	100.0	100.0
43	552	449	81.3	438	97.6	398	88.6	90.9
44	74	69	93.2	55	79.7	47	68.1	85.5
45	96	76	79.2	55	72.4	52	68.4	94.5

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched

Treatment: NP10

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	27	26	96.3	26	100.0	18	69.2	69.2
2	27	11	40.7	11	100.0	8	72.7	72.7
3	3	0	N/A	N/A	N/A	N/A	N/A	N/A
4	142	129	90.8	89	69.0	72	55.8	80.9
5	19	16	84.2	16	100.0	10	62.5	62.5
6	64	62	96.9	61	98.4	42	67.7	68.9
7	64	58	90.6	58	100.0	32	55.2	55.2
8	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
9	14	13	92.9	12	92.3	12	92.3	100.0
10	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12	201	143	71.1	143	100.0	103	72.0	72.0
13	32	28	87.5	25	89.3	22	78.6	88.0
14	146	91	62.3	76	83.5	68	74.7	89.5
15	208	162	77.9	162	100.0	134	82.7	82.7
16	621	497	80.0	477	96.0	398	80.1	83.4
17	35	26	74.3	22	84.6	20	76.9	90.9
18	175	129	73.7	129	100.0	95	73.6	73.6
19	151	118	78.1	113	95.8	87	73.7	77.0
20	437	321	73.5	314	97.8	258	80.4	82.2
21	51	44	86.3	38	86.4	37	84.1	97.4
22	633	466	73.6	376	80.7	287	61.6	76.3
23	484	394	81.4	373	94.7	302	76.6	81.0
24	211	185	87.7	185	100.0	124	67.0	67.0
25	436	278	63.8	278	100.0	229	82.4	82.4
26	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
27	884	779	88.1	650	83.4	554	71.1	85.2

28	176	173	98.3	118	68.2	94	54.3	79.7
29	465	387	83.2	371	95.9	246	63.6	66.3
30	211	177	83.9	177	100.0	137	77.4	77.4
31	564	314	55.7	301	95.9	247	78.7	82.1
32	399	340	85.2	310	91.2	312	91.8	100.6
33	538	449	83.5	419	93.3	387	86.2	92.4
34	392	315	80.4	305	96.8	302	95.9	99.0
35	87	65	74.7	55	84.6	43	66.2	78.2
36	967	859	88.8	800	93.1	687	80.0	85.9
37	618	372	60.2	350	94.1	298	80.1	85.1
38	798	474	59.4	332	70.0	402	84.8	121.1
39	415	330	79.5	325	98.5	285	86.4	87.7
40	68	53	77.9	53	100.0	41	77.4	77.4
41	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
42	270	228	84.4	217	95.2	217	95.2	100.0
43	11	9	81.8	8	88.9	4	44.4	50.0
44	132	98	74.2	62	63.3	37	37.8	59.7
45	99	65	65.7	35	53.8	22	33.8	62.9

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched

Treatment: NP100

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	36	31	86.1	20	64.5	12	38.7	60.0
2	12	10	83.3	8	80.0	5	50.0	62.5
3	248	170	68.5	132	77.6	112	65.9	84.8
4	1	1	100.0	1	100.0	0	0.0	0.0
5	115	65	56.5	61	93.8	47	72.3	77.0
6	103	53	51.5	45	84.9	26	49.1	57.8
7	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	87	71	81.6	68	95.8	52	73.2	76.5
9	2	2	100.0	1	50.0	1	50.0	100.0
10	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
11	47	46	97.9	41	89.1	30	65.2	73.2
12	393	315	80.2	296	94.0	267	84.8	90.2
13	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14	187	140	74.9	69	49.3	47	33.6	68.1
15	486	316	65.0	241	76.3	203	64.2	84.2
16	196	153	78.1	133	86.9	108	70.6	81.2
17	714	600	84.0	569	94.8	498	83.0	87.5
18	273	175	64.1	140	80.0	97	55.4	69.3
19	114	71	62.3	57	80.3	31	43.7	54.4
20	82	61	74.4	37	60.7	35	57.4	94.6
21	262	242	92.4	156	64.5	122	50.4	78.2

22	260	214	82.3	190	88.8	175	81.8	92.1
23	325	270	83.1	183	67.8	143	53.0	78.1
24	179	132	73.7	108	81.8	84	63.6	77.8
25	403	341	84.6	325	95.3	268	78.6	82.5
26	63	52	82.5	48	92.3	30	57.7	62.5
27	117	80	68.4	60	75.0	28	35.0	46.7
28	39	21	53.8	13	61.9	10	47.6	76.9
29	113	93	82.3	57	61.3	52	55.9	91.2
30	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
31	44	8	18.2	4	50.0	3	37.5	75.0
32	28	21	75.0	15	71.4	10	47.6	66.7
33	359	348	96.9	258	74.1	198	56.9	76.7
34	43	38	88.4	38	100.0	32	84.2	84.2
35	18	14	77.8	9	64.3	8	57.1	88.9
36	2	2	100.0	2	100.0	2	100.0	100.0
37	340	281	82.6	266	94.7	205	73.0	77.1
38	63	26	41.3	11	42.3	8	30.8	72.7
39	300	133	44.3	83	62.4	56	42.1	67.5
40	213	172	80.8	160	93.0	124	72.1	77.5
41	143	110	76.9	100	90.9	61	55.5	61.0
42	248	183	73.8	101	55.2	73	39.9	72.3
43	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	46	35	76.1	34	97.1	30	85.7	88.2
45	1	1	100.0	1	100.0	0	0.0	0.0

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched

Treatment: EE1

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	160	102	63.8	70	68.6	66	64.7	94.3
2	179	155	86.6	139	89.7	124	80.0	89.2
3	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	252	224	88.9	224	100.0	215	96.0	96.0
5	202	195	96.5	194	99.5	185	94.9	95.4
6	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	125	102	81.6	102	100.0	95	93.1	93.1
8	107	99	92.5	99	100.0	57	57.6	57.6
9	181	141	77.9	141	100.0	102	72.3	72.3
10	3	2	66.7	2	100.0	0	0.0	0.0
11	91	30	33.0	25	83.3	23	76.7	92.0
12	289	99	34.3	98	99.0	85	85.9	86.7
13	18	13	72.2	12	92.3	10	76.9	83.3
14	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
15	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A

16	132	68	51.5	64	94.1	41	60.3	64.1
17	212	164	77.4	132	80.5	112	68.3	84.8
18	143	30	21.0	30	100.0	24	80.0	80.0
19	119	63	52.9	50	79.4	48	76.2	96.0
20	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
21	102	37	36.3	30	81.1	23	62.2	76.7
22	53	17	32.1	17	100.0	14	82.4	82.4
23	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
25	175	150	85.7	132	88.0	110	73.3	83.3
26	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
27	9	5	55.6	5	100.0	3	60.0	60.0
28	21	9	42.9	7	77.8	7	77.8	100.0
29	764	416	54.5	384	92.3	342	82.2	89.1
30	269	204	75.8	189	92.6	116	56.9	61.4
31	2	0	0	0	N/A	N/A	N/A	N/A
32	113	87	77.0	54	62.1	51	58.6	94.4
33	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
34	148	90	60.8	52	57.8	47	52.2	90.4
35	179	133	74.3	113	85.0	95	71.4	84.1
36	83	56	67.5	41	73.2	40	71.4	97.6
37	486	324	66.7	312	96.3	287	88.6	92.0
38	238	117	49.2	75	64.1	71	60.7	94.7
39	47	40	85.1	40	100	25	62.5	62.5
40	23	17	73.9	16	94.1	16	94.1	100
41	1	1	100	0	0	N/A	N/A	N/A
42	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched

Treatment: EE10

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	48	0	0.0	0	N/A	N/A	N/A	N/A
3	1	1	100.0	0	0.0	N/A	N/A	N/A
4	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	45	15	33.3	9	60.0	5	33.3	55.6
6	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	64	22	34.4	15	68.2	7	31.8	46.7
8	69	26	37.7	12	46.2	4	15.4	33.3
9	143	75	52.4	20	26.7	12	16.0	60.0

10	169	123	72.8	85	69.1	54	43.9	63.5
11	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
12	34	3	8.8	3	100.0	3	100.0	100.0
13	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14	2	0	0.0	0	N/A	N/A	N/A	N/A
15	34	14	41.2	4	28.6	2	14.3	50.0
16	145	97	66.9	78	80.4	32	33.0	41.0
17	66	20	30.3	12	60.0	7	35.0	58.3
18	72	56	77.8	46	82.1	28	50.0	60.9
19	35	26	74.3	26	100.0	15	57.7	57.7
20	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
21	196	182	92.9	153	84.1	94	51.6	61.4
22	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
23	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24	413	270	65.4	227	84.1	124	45.9	54.6
25	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
26	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
27	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
28	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
29	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
30	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
31	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
32	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
33	356	283	79.5	259	91.5	187	66.1	72.2
34	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
35	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
36	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
37	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
38	73	42	57.5	21	50.0	15	35.7	71.4
39	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
40	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
41	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
42	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched

Treatment: EE1+NP10

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	324	247	76.2	244	98.8	202	81.8	82.8
2	44	37	84.1	37	100.0	28	75.7	75.7
3	221	193	87.3	193	100.0	145	75.1	75.1

4	59	49	83.1	47	95.9	42	85.7	89.4
5	104	100	96.2	99	99.0	84	84.0	84.8
6	319	267	83.7	258	96.6	213	79.8	82.6
7	23	21	91.3	20	95.2	17	81.0	85.0
8	75	62	82.7	59	95.2	41	66.1	69.5
9	20	15	75.0	15	100.0	5	33.3	33.3
10	121	107	88.4	105	98.1	67	62.6	63.8
11	35	33	94.3	33	100.0	25	75.8	75.8
12	222	195	87.8	195	100.0	124	63.6	63.6
13	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
15	368	282	76.6	272	96.5	182	64.5	66.9
16	20	18	90.0	18	100.0	8	44.4	44.4
17	34	11	32.4	11	100.0	10	90.9	90.9
18	602	422	70.1	378	89.6	284	67.3	75.1
19	359	231	64.3	203	87.9	178	77.1	87.7
20	73	64	87.7	61	95.3	45	70.3	73.8
21	505	374	74.1	356	95.2	310	82.9	87.1
22	132	98	74.2	87	88.8	43	43.9	49.4
23	155	112	72.3	87	77.7	61	54.5	70.1
24	234	156	66.7	128	82.1	97	62.2	75.8
25	28	23	82.1	17	73.9	11	47.8	64.7
26	90	54	60.0	23	42.6	14	25.9	60.9
27	282	70	24.8	45	64.3	27	38.6	60.0
28	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
29	486	287	59.1	122	42.5	79	27.5	64.8
30	80	14	17.5	13	92.9	7	50.0	53.8
31	169	92	54.4	89	96.7	71	77.2	79.8
32	189	73	38.6	48	65.8	35	47.9	72.9
33	33	20	60.6	18	90.0	11	55.0	61.1
34	380	305	80.3	254	83.3	188	61.6	74.0
35	328	273	83.2	202	74.0	176	64.5	87.1
36	607	472	77.8	335	71.0	284	60.2	84.8
37	202	143	70.8	131	91.6	116	81.1	88.5
38	778	586	75.3	540	92.2	458	78.2	84.8
39	26	16	61.5	15	93.8	14	87.5	93.3
40	2	2	100.0	2	100.0	2	100.0	100.0
41	19	7	36.8	7	100.0	3	42.9	42.9
42	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	797	664	83.3	512	77.1	405	61.0	79.1

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched

Treatment: EE1+NP100

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	102	80	78.4	73	91.3	34	42.5	46.6
2	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	533	355	66.6	323	91.0	218	61.4	67.5
4	212	154	72.6	154	100.0	116	75.3	75.3
5	56	48	85.7	48	100.0	42	87.5	87.5
6	293	253	86.3	249	98.4	205	81.0	82.3
7	1	1	100.0	1	100.0	0	0.0	0.0
8	545	402	73.8	402	100.0	304	75.6	75.6
9	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	98	57	58.2	57	100.0	31	54.4	54.4
11	376	284	75.5	284	100.0	194	68.3	68.3
12	38	33	86.8	31	93.9	17	51.5	54.8
13	37	24	64.9	23	95.8	11	45.8	47.8
14	28	17	60.7	15	88.2	14	82.4	93.3
15	133	55	41.4	40	72.7	18	32.7	45.0
16	197	128	65.0	126	98.4	87	68.0	69.0
17	81	46	56.8	43	93.5	25	54.3	58.1
18	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
19	193	28	14.5	20	71.4	8	28.6	40.0
20	18	5	27.8	3	60.0	1	20.0	33.3
21	589	447	75.9	277	62.0	189	42.3	68.2
22	296	220	74.3	132	60.0	96	43.6	72.7
23	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
24	404	212	52.5	212	100.0	128	60.4	60.4
25	21	3	14.3	3	100.0	3	100.0	100.0
26	287	182	63.4	178	97.8	105	57.7	59.0
27	268	178	66.4	178	100.0	124	69.7	69.7
28	442	284	64.3	221	77.8	211	74.3	95.5
29	340	138	40.6	45	32.6	41	29.7	91.1
30	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
31	781	687	88.0	470	68.4	416	60.6	88.5
32	338	320	94.7	172	53.8	158	49.4	91.9
33	1028	698	67.9	658	94.3	624	89.4	94.8
34	161	8	5.0	8	100.0	6	75.0	75.0
35	272	186	68.4	159	85.5	125	67.2	78.6
36	1272	740	58.2	671	90.7	584	78.9	87.0
37	17	0	0.0	0	N/A	N/A	N/A	N/A
38	436	278	63.8	151	54.3	137	49.3	90.7
39	134	104	77.6	96	92.3	92	88.5	95.8
40	969	789	81.4	781	99.0	629	79.7	80.5
41	444	309	69.6	250	80.9	203	65.7	81.2
42	257	170	66.1	137	80.6	128	75.3	93.4
43	935	778	83.2	740	95.1	641	82.4	86.6

44	122	91	74.6	67	73.6	42	46.2	62.7
45	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched

Treatment: EE10+NP100

Trials	Total Eggs	Fertilized Eggs	% Fertilized	Hatched	% Hatched	Swim-ups	S/E[1]	S/H[2]
1	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
3	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
4	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
5	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8	14	9	64.3	8	88.9	5	55.6	62.5
9	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
10	125	94	75.2	90	95.7	64	68.1	71.1
11	3	2	66.7	2	100.0	2	100.0	100.0
12	9	9	100.0	9	100.0	4	44.4	44.4
13	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14	16	7	43.8	4	57.1	1	14.3	25.0
15	120	77	64.2	69	89.6	47	61.0	68.1
16	112	61	54.5	33	54.1	24	39.3	72.7
17	50	32	64.0	30	93.8	27	84.4	90.0
18	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
19	117	73	62.4	54	74.0	43	58.9	79.6
20	23	12	52.2	12	100.0	8	66.7	66.7
21	92	68	73.9	52	76.5	37	54.4	71.2
22	106	79	74.5	69	87.3	58	73.4	84.1
23	37	24	64.9	23	95.8	18	75.0	78.3
24	40	31	77.5	27	87.1	22	71.0	81.5
25	183	134	73.2	102	76.1	84	62.7	82.4
26	5	2	40.0	1	50.0	0	0.0	0.0
27	106	71	67.0	71	100.0	52	73.2	73.2
28	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
29	76	42	55.3	40	95.2	28	66.7	70.0
30	101	53	52.5	47	88.7	32	60.4	68.1
31	92	75	81.5	72	96.0	61	81.3	84.7
32	37	17	45.9	13	76.5	9	52.9	69.2
33	18	12	66.7	12	100.0	9	75.0	75.0
34	117	52	44.4	43	82.7	31	59.6	72.1
35	138	111	80.4	65	58.6	48	43.2	73.8
36	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
37	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A

38	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
39	5	0	0.0	N/A	N/A	N/A	N/A	N/A
40	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
41	66	54	81.8	53	98.1	44	81.5	83.0
42	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
43	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
44	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A
45	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[1]S/E = ratio of # of swim-ups to # of fertilized eggs
[2]S/H = ratio of # of swim-ups to # hatched